8-9-2019

Investigating the Role of Neuraminidase Activity in the Co-pathogenesis of Mycoplasma gallisepticum and Low Pathogenic Avian Influenza A Virus

Jessica Canter
University of Connecticut - Storrs, jessica.canter@uconn.edu

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Jessica A. Canter, PhD

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Abstract

The avian pathogen *Mycoplasma gallisepticum*, the etiologic agent of chronic respiratory disease in chickens, exhibits enhanced pathogenesis in the presence of a co-pathogen such as low-pathogenic avian influenza virus (LPAIV). *M. gallisepticum* R_{low} can utilize α-2,3 linked sialic acids, abundant in the avian respiratory tract, to bind host cells *in vitro*. To further investigate the intricacies of this co-pathogenesis, chickens were mono- or co-infected with either virulent *M. gallisepticum* strain R_{low}, attenuated *M. gallisepticum* neuraminidase mutant P1C5 and Mycoplasma specific lipoprotein A mutant P1H9, or LPAIV H3N8 (A/duck/Ukraine/1963). These chickens were then assessed for tracheal histopathology, pathogen load, and transcriptomic host response to infection using RNA-sequencing. Chickens co-infected with *M. gallisepticum* R_{low} followed by H3N8 exhibited significantly more severe tracheal lesions and mucosal thickening in response to infection than chickens infected with H3N8 alone. Viral load was also significantly increased in this group over chickens who were infected first with H3N8 and subsequently with *M. gallisepticum* R_{low}. The attenuated *M. gallisepticum* mutants P1C5 and P1H9, previously shown to be cleared 14 days post-infection, were able to persist 6 to 7 days post-infection in the presence and absence of co-infection

with H3N8. P1H9 was able to persist to 14 days post-infection only in the presence of H3N8. The transcriptional response to mono- and co-infection with *M. gallisepticum* and LPAIV highlighted the involvement of differential expression of genes such as TLR4, TLR15, TLR21, IL-1β, IRF4, MMP1, and MMP9. Pathway and gene ontology analysis of these differentially expressed genes suggests that co-infection with virulent *M. gallisepticum* and LPAIV induces a downregulation of ciliary activity *in vivo* and alters the multiple immune-related signaling cascades. Although H3N8 is susceptible to neuraminidase inhibition by oseltamivir *in vitro*, this antiviral treatment was not effective *in vivo* at reducing H3N8 load in the trachea of co-infected chickens. These data indicate that the co-pathogenesis of LPAIV and *M. gallisepticum* is not strictly neuraminidase-dependent and warrants further experimental understanding.

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Jessica A. Canter

B.S., Towson University 2012

M.S., Towson University 2015

A Dissertation Submitted
In Partial Fulfillment of the
Requirements for the Degree
Of Doctor of Philosophy
At the University of Connecticut

August 2019

APPROVAL PAGE

Doctor of Philosophy Dissertation

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Presented by
Jessica A. Canter, B.S., M.S.

Co-Major Advisor _____

Steven J. Geary

Co-Major Advisor _____

Steven M. Szczepanek

Associate Advisor _____

Meghan A. May

Associate Advisor _____

Dong-Hun Lee

University of Connecticut, 2019

Acknowledgements

I would first like to thank my major advisors, Dr. Steven Geary an Dr. Steven Szczepanek for their constant support and guidance throughout my degree process. The members of my advisory committee, Dr. Meghan May and Dr. Dong-Hun Lee, also deserve my sincerest thanks for their perspective on experimental design, data analysis, and overall assistance.

I would also like to extend my gratitude to the members of the Geary Lab, current and former, for not only giving me the tools to succeed scientifically, but the support of friendship as well. Edan Tulman and Xiaofen Liao were instrumental in providing great scientific and technical advice and a welcoming, collegial environment. My fellow graduate students Katie Pflaum, Jessica Beaudet, and Arlind Mara were absolutely essential in sharing their skills and experiences during experiments and steadfast friendship. My thanks also go to my fellow graduate students of the Pathobiology and Veterinary Sciences department here at UConn for their comradery and support as well as the faculty and staff in the department for their helpful feedback during my education.

Finally, I would like to extend my deepest thanks to my parents, Donald and Christine Canter, for their unwavering love and assistance throughout this process. The two of them have always set a great example for the value of hard work. I truly owe all of my successes to their love and support.

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Section 1 – Broad Introduction to Mycoplasmas

History and Nomenclature of Mycoplasmas

In 1898 an infectious organism, described at the time as a "virus", was isolated from clinical samples taken from a cow stricken with contagious bovine pleuropneumonia [1]. This organism was filterable but not entirely invisible and had also become associated with mastitis, polyarthritis, and agalactia in goats and sheep [2]. Over the coming decades, there was little to no consensus on the nomenclature of these agents of disease. The umbrella term of "pleuropneumonia-like organisms" (PPLO) was employed to encompass this and related organisms [3].

It was not until 1955 that the term "Mycoplasma" became formally used to identify these organisms within the order *Mycoplasmatales* [4] and the genus *Mycoplasma* [5]. In the following decade, a Subcommittee on the Taxonomy of Mycoplasma determined that the class *Mollicutes* should thus be characterized by the lack of a true cell wall and a plastic outer membrane [5,6]. It was around this same era that the study of *Mycoplasmas* began in earnest with the determination that Eaton's agent, the cause of primary atypical pneumonia in humans, was classified as a *Mycoplasma* [8,9].

General Mycoplasma Characteristics

Although the lack of a classical prokaryotic cell wall became an integral aspect of the identity of *Mollicutes* early on [6,7] there are other crucial elements to the unique nature of *Mycoplasmas*. These organisms are among the smallest, self-replicating prokaryotes with a highly reduced genome size ranging between 580 kB in the human pathogen *Mycoplasma genitalium* [10] to 1380 kB in the bovine pathogen *Mycoplasma mycoides* subsp. *mycoides* [11].

These small genomes are also comprised of a low guanine/cytosine content [11] and are prone to mutation because of poor DNA polymerase proofreading [12]. Another feature of *Mycoplasmas* are their limited biosynthetic abilities as a byproduct of their reduced genomic size. This warrants the need for an intimate relationship between *Mycoplasmas* and their host environment for the acquisition of molecules and nutrients essential for life [11]. Although this host-microbial relationship is critical, it is not limited by a narrow host organism range nor always a pathogen-host dynamic [11].

Innate Immune Response to Mycoplasmas

The innate host response to *Mycoplasmas* begins at the mucosal interface. These mucosal barriers serve as the first line of defense in the host innate immune system. *Mycoplasmas* of the respiratory tract, for instance, interact with cilia in the host respiratory epithelium at sialo-oligosaccharide receptors [13]. Lipoproteins on the surface of *Mycoplasmas* are sometimes involved in this interaction and are sensed by

pattern recognition receptors such as toll-like receptors (TLRs) and NOD-like receptors (NLRs) [14].

These mycoplasma lipoproteins most notably are recognized by TLRs 1, 2, and 6 [14]. These TLRs all serve to recognize pathogen-associated molecular patterns (PAMPs) in the form of bacterial lipoproteins [14]. These particular TLRs have a concerted relationship in that TLR 2 interacts with TLRs 1 or 6 to bind both tri- and di-acetylated lipoproteins [15,16]. These lipoproteins are key in *Mycoplasmas* such as *Mycoplasma pulmonis*, which uses its family of Vsa lipoproteins in evasion of phagocytosis by host alveolar macrophages [17,18].

Mycoplasma lipoproteins, such as those from *Mycoplasma fermentans* and *Mycoplasma salivarium* are associated with activation of the inflammasome of the host [19]. *Mycoplasma genitalium* is also recognized by TLRs 2 and 6 in the host, activating NFκB and leading to downstream induction of interleukin (IL) 6, IL-7, IL-8, monocyte chemoattractant protein 1 (MCP1) early in infection [20,21]. Other notable instances of *Mycoplasma* lipoproteins involved in pathogenesis and immune evasion will be discussed in later portions of this dissertation among a wide range of hosts.

The activation of the host inflammasome can also be mediated by extracellular ATP production and macrophage stimulation, as seen in *Mycoplasma hominis,* which subsequently leads to IL-1β production contributing to the pro-inflammatory immune response [19,22]. The inflammasome can also be stimulated by *Mycoplasma pneumoniae* exotoxin, community-acquired respiratory distress (CARDS) toxin, by its ADP-ribosylation activity [23]. *M. pneumoniae* has also been shown to induce IL-8, IL-1β, tumor necrosis factor (TNF) α, and regulated on activation, normal T cell expressed

and secreted (RANTES) [24]. Other pathogens, such a *Mycoplasma hyopneumoniae* increase the expression of IL-5 and IL-13 in the host [25].

Mycoplasmas have also been documented to induce the release of neutrophil extracellular traps (NETs). NETs are released by neutrophils in response to microbial infection and consist of chromatin, cytoplasmic proteins, antimicrobial peptides, and enzymes to neutralize microbial pathogens [26,27]. This release of NETs from the cytoplasm of neutrophils is classified as NET-osis, or a variety of cellular death [26]. Infection with *Mycoplasma bovis* stimulates reactive oxygen species production and the later release of these NETs [28]. *Mycoplasmas* can also inhibit cell cycle checkpoints by modulating p53 and cellular apoptosis [29].

Another facet of host immunity relevant to *Mycoplasma* infection is the family of matrix metalloproteinases (MMPs). These MMPs are produced by an array of immune cells such as macrophages and T cells in response to cytokine stimulation and contribute to tissue remodeling and inflammation mediation [30]. *Mycoplasma hyorhinis*, for instance, activates MMP-2 with its antigen p37 which can contribute to carcinogenesis [31]. MMP-2, as well as MMP-9 are increased in expression in response to infection with *Mycoplasma pulmonis* in the respiratory tract [32]. *Mycoplasma pneumoniae* also induces MMP-9 in human circulation during infection of the airway [33]. The innate immune response to *Mycoplasmas* will be further described throughout this dissertation with a focus on response to infection with *Mycoplasma gallisepticum*.

Mycoplasma Sialidases (Neuraminidases)

Sialidases, also referred to as neuraminidases, are glycosyl hydrolase enzymes present in prokaryotes, eukaryotes, and viruses [34]. These sialidases release terminal *N*-acetylneuraminate, also known as sialic acid, residues from glycoproteins, glycolipids and polysaccharides [34]. Sialic acids are nine-carbon sugars that exist in four different categories: *N*-acetylneruaminic acid (Neu5Ac, NANA) which is most relevant to vertebrates, *N*-glycolneuraminic acid (Neu5Gc), deaminated neuraminic acid (KDN), and neuraminic acid (Neu) [34]. These sialic acids can have an array of modifications at the hydroxyl group position that can influence recognition by sialic acid binding proteins [34]. Linkages of sialic acids to galactose molecules on the cell surface are commonly seen in α(2,3)-, α(2,6)-, and α(2,8)- confirmations [34].

Bacteria can utilize sialidase activity to recognize host cells, colonize and disseminate within the host, degrade the extracellular matrix and scavenge sialic acids as nutrients [35,36,37,38]. Certain species, such as *Streptococcus pneumoniae,* have specific sialidase activity for only α(2,3)-linked sialic acids [34]. Other organisms, such as *Actinobacter urefaciens* utilize sialidase to hydrolyze α(2,3)-, α(2,6)-, and α(2,8)-linked sialic acids [34]. In many bacterial genomes, the gene for sialidase is often located in close proximity to genes encoding enzymes facilitating the transport and intracellular metabolism of sialic acids and their byproducts for glycolysis [36].

Sialidase activity is frequently associated with bacterial virulence. For instance, the sialidase gene *nanA* in *Streptococcus pneumoniae* is critical for virulence in the nasopharyngeal tract of the host and both *nanA* and *nanB* sialidase genes are associated with upper and lower respiratory tract infections and sepsis [39]. The first

Mycoplasma with reported sialidase activity was the avian pathogen *Mycoplasma gallisepticum* [40,41,42]. The activity of sialidase varies widely among *Mycoplasma* species [43,44].

Notably, another avian *Mycoplasma* pathogen, *Mycoplasma synoviae*, retains the genes for sialic acid degradation and metabolism in its genome [45], whereas *Mycoplasma gallisepticum* does not [46]. It is hypothesized that these genes were transferred between these two species and that *M. gallisepticum* has lost the sialic acid metabolism genes over time [47]. A more detailed discussion of sialidase in different *Mycoplasma* species can be found in the subsequent review of pathogenic *Mycoplasmas* and their hosts. There is evidence that sialidases from co-infecting pathogens, i.e. a viral and bacterial pathogen, may have a synergistic relationship contributing to the enhancement of disease. Instances of this pathogenic phenomenon will also be discussed later in this dissertation.

Sub-Section 1-1: Pathogenic Mycoplasmas

Mycoplasmas of Mammals - Humans

Humans play host to multiple Mycoplasmal pathogens in both the respiratory and reproductive tract. *Mycoplasma pneumoniae*, formerly known as Eaton's agent [8] is responsible for 4-8% of all community acquired bacterial pneumonia cases [48]. This proportion can increase to 20-40% during an endemic and 70% within a closed population such as a household or school [48]. *Mycoplasma pneumoniae* is readily spread within a closed community, such as a family unit, and has an incubation period of 23 days [49].

The atypical or "walking" pneumonia, caused by *M. pneumonia* is characterized by tracheobronchitis, a cough either with or without sputum, and general malaise [50]. Infection with *M. pneumoniae* becomes even more serious in patients with chronic asthma or chronic obstructive pulmonary disorder (COPD) due to dysregulation of host inflammatory responses [51]. This pathogen can migrate to extrapulmonary sites in the host causing encephalitis in 13% of cases, as well as stroke and psychological disorders, dermatological pathology, gastrointestinal symptoms, carditis, and septic arthritis [48,52]. Natural immunity to *M. pneumoniae* is not long-lived and a patient will shed the pathogen over a chronic period [48].

One unique feature of *M. pneumoniae* is the presence of an endotoxin named the community-acquired respiratory distress (CARDS) toxin [53,54]. This endotoxin has ADP-ribosylation activity and its expression is induced when *M. pneumoniae* encounters a host [54]. This association with the host respiratory tract requires the cytadherance

and gliding motility of *M. pneumoniae* mediated by the adhesin molecules P1, P90, P40 and P30 which assemble into an attachment organelle structure [55].

Another human respiratory pathogen is *Mycoplasma amphoriforme* which was originally isolated in 1999 from a patient with x-linked agammaglobulinemia and chronic bronchitis [56]. It has since been isolated from immunocompromised patients with respiratory tract infections in Europe [56,57]. Similar to *M. pneumoniae*, *M. amphoriforme* also utilizes an attachment organelle structure in cytadherance and has gliding motility [58].

In addition to the respiratory tract, *Mycoplasmas* are also associated with disease in the human urogenital tract. One such pathogen is *Mycoplasma genitalium* which was first detected in 1981 in male patients with non-gonococcal urethritis [59]. *M. genitalium* has since been associated with urethral inflammation and discharge in male patients and cervicitis with chronic persistence in female patients [60]. The *M. genitalium* cell has a flask-shaped morphology and has a similar attachment organelle to *M. pneumoniae* that is mediated by the proteins MgpB and MgpC [61,62]. These attachment proteins also experience genetic variation by recombination [63,64].

Another noteworthy urogenital pathogen is *Mycoplasma hominis* which is associated with pelvic inflammatory disease (PID), bacterial vaginosis, spontaneous abortion, and neonatal bacteremia and meningitis [65,66]. The pathobiology of *M. hominis* is complicated by aberrant inflammatory cytokine signaling from pregnant mother and fetal hosts which ultimately leads to pre-term birth and fetal disease in affected patients [67]. *M. hominis* also possesses variable adherence associated (Vaa) antigens which are phase variable and immunogenic in the human host [68].

Other relevant human *Mycoplasma* pathogens include *Mycoplasma penetrans*, which employs an attachment organelle and gliding motility to cause disease in the urogenital tract [69]. *M. penetrans* is strongly associated with disease in patients with acquired immunodeficiency syndrome (AIDS) [70]. Lastly, *Mycoplasma fermentans* is a urogeneital *Mycoplasma* implicated in genital tract infections, genital ulcers, and also rheumatoid disease and respiratory infections [71,72].

Mycoplasmas of Mammals - Swine

There are two predominant *Mycoplasma* pathogens of porcine species: *Mycoplasma hyopnumoniae* and *Mycoplasma hyorhinis*. The first pathogen, *M. hyopneumoniae*, is responsible for enzootic pneumonia and a member of the porcine respiratory disease complex (PRDC) [73]. This makes *M. hyopneumoniae* of great relevance and concern to the agricultural industry. Infected pigs are susceptible to co-infection with other respiratory pathogens, as will be discussed later in this review, and can be exposed to *M. hyopneumoniae* through lactation [74]. *M. hyopneumoniae* uses an array of cytadhesins (P97, P102, P159, and P146) to bind the pig respiratory tract resulting in ciliostasis and ciliary loss in these animals [75,76].

Another *Mycoplasma* pathogen of swine is *Mycoplasma hyorhinis* which is associated with polyserositis and polyarthritis in nursing piglets [Kobish and Friis, 1996]. This pathology results in reduced weight gain in piglets as they age and lameness, making *M. hyorhinis* infection another agriculturally relevant *Mycoplasma* [77. *M. hyorhinis* employs a family of variable lipoproteins (Vlp) such as VlpA, VlpB, and VlpC which serve as variable surface antigens [77]. In addition, *M. hyorhinis* has been

recently associated with extracytoplasmic binding via P37, which can induce the migration of cancer cells by n-terminal annexin A2, leading to tumor progression [78].

Mycoplasmas of Mammals - Bovids

In cattle, *Mycoplasma bovis* is a pathogen of great economic relevance to the agricultural industry as it is the most common cause of Mycoplasma mastitis in cows [79]. This pathogen is also an agent of chronic pneumonia and poly-arthritis syndrome (CPPS) and bovine respiratory disease (BRD) in cattle [80,81]. Infection with *M. bovis* is often associated with co-infection in immunosuppressed animals afflicted with viral infections such as bovine diarrheal virus and bovine herpes virus 1 [82,83]. As with other notable *Mycoplasma* pathogens, *M. bovis* also utilizes a family of variable surface proteins (Vsp) that are involved in attachment and host-pathogen recognition [84].

There is also a group of closely related *Mycoplasma* species that infect bovine or caprine hosts denoted as the *Mycoplasma mycoides* Mycoides cluster [85]. This cluster consists of one bovine pathogen, *M. mycoides* subsp. *mycoides* Small Colony (SC), and four caprine pathogens; *M. mycoides* subsp. *capri* (Mmc), *M. capricolum* subsp. *capricolum* (Mcc), *M. capricolum* subsp. *capripneumoniae* (Mccp), and *M. leachii* (Ml) [85,86]. Previously, *M. mycoides* subsp. *mycoides* Large Colony (LC) was a member of this cluster, however it has since been classified as serovars within Mmc [86]. The evolution of this cluster of pathogens coincides with the historical domestication of ruminants by humans [87].

The first pathogen in this cluster, *M. mycoides* subsp. *mycoides* SC is the causative agent of contagious bovine pleuropneumonia (CBPP) [87,88]. This disease is classified by respiratory disease with extremely high mortality in infected animals as well as weight loss, reduction in fertility, and crippling economic impact to the cattle industry from quarantine and disease control measures [88]. In Africa, the Pan African Control of Epizootics (PACE) has declared CBPP the second most important transboundary disease in Africa, preceded only by Rinderpest [88]. *M. mycoides* subsp. *mycoides* SC is the only bacterial pathogen to be classified as an OIE List A pathogen [89].

Infected cattle can be either symptomatic or asymptomatic carriers of the disease, and this infection can transition to a chronic form over time [88]. The immune response to *M. mycoides* subsp. *mycoides* SC is characterized by an overly robust inflammatory response in the lung which sets the stage for mortality by respiratory distress in as many as 30% of infected cattle [89]. This pathogen is equipped with surface lipoproteins LppA, LppB, and LppQ as well as a family of phase-variable surface proteins (Vmm), all of which are involved in the pathogenesis of CBPP [88,90].

Mycoplasmas of Mammals – Caprids

As mentioned previously, a portion of the *Mycoplasma mycoides* Mycoides cluster are pathogens of caprine species. To begin, *M. mycoides* subsp. *capri* (Mmc) is associated with systemic disease in caprine species known as mastitis, arthritis, keratitis, pneumonia and septicemia (MAKePS) syndrome [91,92]. Another Mycoides cluster pathogen, *M. capricolum* subsp. *capricolum* (Mcc), is responsible for contagious

agalactia and is the foremost cause of this disease in caprine species in France and Spain and effects goat populations worldwide [93].

Lastly, *M. capricolum* subsp. *capripneumoniae* (Mccp) is the pathogen responsible for contagious caprine pleuropneumonia (CCPP) in goats, sheep and wild ruminants [92]. Mccp, formerly known as *Mycoplasma* biotype F38, induces a serofibrinous pleupneumonia and ciliostasis in the respiratory tract with approximately 100% morbidity and mortality [94]. The disease process is often more acute in young animals and chronic in older animals [94]. Currently, it can be detected in over 40 countries worldwide with the exception of those on the American continent [95]. As with other members of the Mycoides cluster, Mccp is of great concern to the agricultural industry causing over $507 million dollars in losses per year in endemic areas [95].

Outside of the Mycoides cluster, *Mycoplasma agalactiae* is a pathogen of sheep and goats causing contagious agalactia [94]. *M. agalactiae* can be transmitted vertically through milk feeding [94]. This pathogen also carries immunodominant surface lipoproteins (Vpma) that are involved in pathogenesis in the host [96]. Lastly, *Mycoplasma ovipneumoniae* is a common pathogen of sheep, effecting approximately 90% of sheep operations in the United States [97]. It is also the primary cause of epizootics of pneumonia in bighorn sheep [98]. *M. ovipneumoniae* induces interstitial pneumonia and is often detected in animals co-infected with pathogens such as *Mannheimia haemolytica* [99].

Mycoplasmas of Mammals – Felines

Mycoplasma haemofelis is a hemotropic Mycoplasma, also known as a Hemoplasma, that binds to feline red blood cells and can cause severe hemolytic anemia in wild and domestic cats [100]. This infection state can be acute or can transition to a chronic carrier state when infected cats receive antibiotic treatment and later re-activate *M. haemofelis* infection [101]. Other feline pathogens include *Mycoplasma felis*, which causes upper respiratory tract infections, and *Mycoplasma gatae*, which is associated with arthritis and tenosynovitis [102,103].

Mycoplasmas of Mammals – Canines

Mycoplasma canis is an opportunistic pathogen of dogs which otherwise resides as a commensal organism in the respiratory or urogenital tract [104,105]. It has been found in the brains of dogs exhibiting granulomatous meningoencephalitis and necrotizing meningoencephalitis [104]. Another pathogen, *Mycoplasma cynos*, is a respiratory pathogen first isolated from a fatal infection in a puppy [106]. *M. cynos* employs the hemagglutinin HapA in cytadherance to host cells [106]. In addition, both *M. canis* and *M. cynos* are positive for sialidase activity as well as the other canine-associated *Mycoplasma edwardii, Mycoplasma spumans,* and *Mycoplasma molare* [107].

Mycoplasmas of Mammals – Rodents

The respiratory pathogen, *Mycoplasma pulmonis*, is often detected in animal care facilities in rodents and is the etiologic agent of murine respiratory mycoplasmosis (MRM) and arthritis [108]. This disease can be transmitted both vertically and horizontally, although different strains of laboratory rodents have altered susceptibility to *M. pulmonis* infection [108,109]. Recently, evidence suggests that IL-17A exacerbates *M. pulmonis* disease in Balb/c mice [109].

Another rodent pathogen is *Mycoplasma arthiditis* which possesses the *Mycoplasma arthritidis*-derived mitogen (MAM) superantigen [110]. *M. arthriditis* is associated with arthritis, toxic shock and necrotizing fasciitis in rodent species [111,112]. Additionally, *Mycoplasma neurolyticum* is associated with neurologic pathology in young mice and rats and possesses weak sialidase activity [113].

Mycoplasmas of Aquatic Vertebrates

The host range of *Mycoplasmas* are not limited to mammalian species. For instance, *Mycoplasma agassizii* is the causative agent of upper respiratory tract disease (URTD) in Mojave Desert tortoises, North American tortoises and gopher tortoises [114,115].

There are two other notable pathogenic *Mycoplasmas* of reptiles. The first of which is *Mycoplasma alligatoris* which is associated with invasive, multisystemic inflammatory disease in alligators and caimans [116,117]. This pathogen has both hyaluronidase and sialidase activity and can scavenge *N*-acetylneuraminate for

catabolism [116,117,118]. *M. alligatoris* also induces apoptosis in host cells by promoting CD95, or FasR, expression in fibroblasts [119].

A closely related pathogen of reptiles is *Mycoplasma crocodyli*, first isolated in 1997 from the joints and lungs of a crocodile [117,120]. In contrast to *M. alligatoris*, *M. crocodyli* is associated with less invasive disease such as mild pneumonia or polyarthritis [117]. Another key difference between these two pathogens is that *M. crocodyli* is free of any sialidase or *N*-acetylneuraminate catabolism genes, although it does have hyaluronidase activity [117]. This pathogen also has no identified adhesin or variable surface antigen genes [117].

Another pathogen of aquatic hosts is *Mycoplasma mobile* which is responsible for necrosis in the gill organs of freshwater fish [121]. Like some *Mycoplasmas* of mammalian hosts, *M. mobile* possesses gliding motility and data suggests that *M. mobile* has the fastest motility among the *Mycoplasmas* [122]. This pathogen also employs mobile variable surface proteins (Mvsp) as variable surface antigens [123].

Mycoplasmas of Avians

There are many *Mycoplasma* pathogens of various avian species. *Mycoplasma meleagridis* is a pathogen of turkeys which causes stunted growth, airsacculitis and decreased egg production [124]. Similar to other pathogenic *Mycoplasmas*, *M. meleagridis* is positive for sialidase activity [113]. Another sialidase positive pathogen is *Mycoplasma corogypsi* which causes polyarthritis and tenosynovitis in black vultures [113,125].

In waterfowl, *Mycoplasma cloacale, Mycoplasma anatis*, and *Mycoplasma anseris* have been associated with airsacculitis, nervous system disease and reproductive disease [126]. *Mycoplasma gallinarum* is responsible for fatty liver hemorrhagic syndrome in commercial layer chickens and can also impact egg quality [127]. Another avian pathogen with relevance to reproductive disease is *Mycoplasma iowae* which can colonize avian embryos as well as reside within the gastrointestinal tract [128]. The pathogenesis of *M. iowae* is controlled by exposure to atmospheric oxygen impacting activity of its catalase, KatE [129]. Also of note, *M. iowae* carries a homologous gene to the *Mycoplasma pneumoniae* CARDS toxin [130].

In wild birds, *Mycoplasma sturnii* is often isolated from conjunctival lesions alongside the pathogen *Mycoplasma gallisepticum*, which will be discussed in greater detail in a later section of this review [131,132]. However, *M. sturnii* does not cause disease when used as the lone inoculum of experimentally infected house finches [132]. Lastly, *Mycoplasma synoviae* causes osteoarthritis, respiratory lesions and synovitis in landfowl [133].

Infection with *M. synoviae* is often accompanied by co-infecting pathogens such as Newcastle disease virus, infectious bronchitis virus, *Escherichia coli*, *Mycoplasma gallisepticum*, or *Mycoplasma meleagridis* [134,135]. As with other notable *Mycoplasmas* mentioned, *M. synoviae* is also positive for sialidase activity and possesses the genes required for sialic acid catabolism which may have been transferred from *Mycoplasma gallisepticum* [47,135].

Spiroplasmas and Phytoplasmas of Plants, Insects, and Invertebrates

Within the *Mollicutes*, there are also relevant pathogens such as *Spiroplasmas* and *Phytoplasmas*. In the 1980's, *Spiroplasmas* were first isolated from crops stricken with citrus stubborn disease and the surfaces of plants [136]. These organisms are also associated with arthropod and crustacean hosts, likely using them as a reservoir host with horizontal transfer between these hosts occurring through interaction with infected plants [137]. Notable example species of these *Spiroplasmas* include *S. apis, S. floricola,* and *S. citri* [138].

Phytoplasmas, on the other hand, are limited to plant hosts and were first characterized in 1967 from plants effected by Yellow's disease [139]. Although *Phytoplasmas* were initially implicated in plant disease, they can also result in ornamental flora manifestation in host plants such as poinsettias [140]. These pathogens are uncultivable outside of the host [138].

Sub-Section 1-2: Mycoplasma gallisepticum

Significance

Mycoplasma gallisepticum is an avian pathogen of great agricultural relevance and will be the *Mycoplasma* of interest for this dissertation. This pathogen is an etiologic agent of chronic respiratory disease (CRD) in chickens, which is characterized by coughing, sneezing, respiratory rales, and nasal and ocular discharge [141,142,143]. The respiratory pathology of *M. gallisepticum* infection is composed of tracheal lesions, airsacculitis, squamous cell metaplasia, ciliary loss in the respiratory epithelium, and thickening of the tracheal mucosa [144].

Within a flock, *M. gallisepticum* is readily transmitted by horizontal transmission via respiratory droplets or conjunctival exposure [145,146], as well as vertical transmission via egg laying [147]. The easy spread of *M. gallisepticum* necessitates culling of infected flocks to contain the infection, as well as losses in egg production and egg quality [148,149]. It has been estimated that *M. gallisepticum* is responsible for $588 million dollars in yearly economic losses in the broiler chicken industry and $132 million dollars yearly in the egg industry [150,151].

In addition to chickens, *M. gallisepticum* is a pathogen of other avian species. This pathogen causes sinusitis in turkeys and conjunctivitis in wild passerines such as the house finch [11,152]. Transmission of *M. gallisepticum* readily occurs within populations of wild birds in wildlife rehabilitation facilities and is impacted by the density of bird feeders in outdoor environments [153,154]. Notably, *M. gallisepticum* appears to exhibit marked host adaptation over time. The *M. gallisepticum* strain isolated from

house finches in 1996 (Virginia 1994, VA94) induces severe pathology in chickens, whereas a more house finch adapted isolate (Virginia 2013, VA13) is attenuated in the chicken host [155].

Efforts to control *M. gallisepticum* infection began as early as the 1960's when the United States federal government instituted its National Poultry Improvement Plan (NPIP) [156]. More recently, *M. gallisepticum* is among the top three avian pathogens of concern in the United States as stated by the USDA Agricultural Research Service (ARS) and Cooperative State Research, Education, and Extension Service (CSREES) [157].

Over time there have been many iterations of vaccination strategies against *M. gallisepticum*. Bacterins (inactive suspensions of whole *M. gallisepticum* cells) showed promise early on, however further study suggested that this vaccine was not efficacious in reducing bacterial load or infection with related *M. gallisepticum* strains [158,159,160,161]. The addition of an adjuvant, such as chitosan, does appear to enhance protection in layer hens [162].

Live attenuated *M. gallisepticum* vaccines are a much broader area of vaccine research. The attenuated F strain of *M. gallisepticum* elicits a robust serological response in vaccinated birds, however this response is not as strong as a natural infection with virulent *M. gallisepticum* [163,164]. F strain has also been associated with respiratory lesion formation in turkeys and young chickens which limit its appeal as a vaccine [165]. Overall, vaccination through the ocular route is more efficacious than the nasal route with the F strain vaccine MycoF [166].

The temperature-sensitive *M. gallisepticum* mutant ts-11 has been shown to be a safer vaccine candidate than F strain, but with reduced immunogenicity [167]. Similarly, the *in vitro* passage attenuated strain 6/85 is a fairly safe vaccine candidate but also is less efficacious [168]. The systemic antibody response to ts-11 is acutely strong and wanes over time, whereas the response to 6/85 is initially weak and slowly increases over time 169]. In recent years two laboratory-generated attenuated *M. gallisepticum* variants, GT5 and Mg7, have undergone study as promising vaccine candidates [170,171,172,173].

Virulence Factors

There is an array of virulence factors that have been characterized in *Mycoplasma gallisepticum*. The sequencing of the virulent *Mycoplasma gallisepticum* strain R_{low} genome was instrumental in outlining and describing some of these virulence factors [46].

A subset of genes are lost after serial *in vitro* passage of the virulent, low passage R_{low} strain to its attenuated, high passage R_{high} strain. These genes are two cytadhesins, GapA and CrmA, and one the high-affinity transporter HatA [174,175]. GapA and CrmA have homology to *Mycoplasma pneumoniae* adhesins [173,174]. Complementation of the attenuated R_{high} with both GapA and CrmA restores cytadherance and virulence *in vivo*, whereas complementation with only GapA results in an attenuated variant, GT5, which has diminished cytadherance properties [174,175]. Other adhesion related proteins include Hlp3 and PlpA, which also share homology with

adhesins of *M. pneumoniae*, and are capable of binding the gelatin and heparin binding domains of fibronectin [176].

Metabolism-related factors also contribute to *M. gallisepticum* virulence. The dihydrolipoamide dehydrogenase (*lpd*) gene within the pyruvate dehydrogenase pathway contributes to the production of ATP in glycolysis [171]. Transposon inactivation of *lpd* results in a metabolically weakened variant, Mg7, which is attenuated in chickens and a promising vaccine candidate [171]. The ABC sugar transport permease MalF is also a factor required for proper metabolism of *M. gallisepticum* and transposon inactivation of MalF ablates *M. gallisepticum* persistence *in vivo* [177]. Conversely transposon mutants in the glycerol transport and hydrogen peroxide production pathway, GlpO, GlpK, and GlpF, retain their virulent phenotype *in vivo* [178]. Some, but not all, *M. gallisepticum* vaccine strains do not produce hydrogen peroxide so the role of this pathway in *M. gallisepticum* is not fully understood [178].

As mentioned previously in this dissertation, sialidases are known to be bacterial virulence factors. Sialidase, or neuraminidase, activity had been described in some strains of *M. gallisepticum* historically, but its role was not deeply explored [41,179]. The *M. gallisepticum* R_{low} gene MGA_0329 was identified as a sialidase with robust activity [46,180]. Transposon insertional inactivation of this gene in three *M. gallisepticum* mutants results in a loss of sialidase activity, persistence, and reduction of virulence *in vivo* compared to virulent R_{low} [180]. One such *M. gallisepticum* mutant, P1C5, was complemented with MGA_0329 and this complementation successfully restored sialidase activity but not virulence or persistence in chickens 14 days post-infection

[180]. These data suggest that the relationship between *M. gallisepticum* sialidase and its virulence in the host is not yet fully elucidated.

Another virulence factor within *M. gallisepticum* R_{low}, MGA_0674, has been characterized as *Mycoplasma*-specific lipoprotein A (MslA) [181]. The *mslA* gene has paralogues in *Mycoplasma pneumoniae* and homologs in other *Mycoplasma* species [181]. MslA is immunogenic and differentially expressed between the virulent strain R_{low} and the attenuated F strain [181]. Transposon insertional inactivation of *mslA* yielded the mutant P1H9, which cannot persist nor cause disease in chickens 14 days post-infection [181]. Evidence indicates that MslA has polynucleotide binding activity for ssDNA, dsDNA, and ssRNA and may use this binding activity to scavenge and utilize these nucleotides [182].

A family of genes, the variable lipoprotein hemagglutinin A (*vlhA*) genes, have been described in both chicken and passerine *Mycoplasma gallisepticum* strains [46,183]. The expression of these *vlhA* genes appears to be coordinated, and changes over time in the tracheae of infected chickens [155,184]. One *vlhA* in particular, *vlhA 3.03*, is the predominantly expressed vlhA in both virulent and attenuated *M. gallisepticum* variants *in vivo*, early in infection [155,184]. However, transposon inactivation of *vlhA 3.03* does not diminish virulence and the expression pattern of the remaining *vlhA* genes remains unchanged from its wild-type counterpart [185]. Two of these VlhA's, VlhA 1.07 and VlhA 4.01, alongside GapA and PlpA and others are considered to be immunogenic, *in vivo i*nduced antigens in chickens [186]. Overall, the role of VlhA expression in virulence of *M. gallisepticum* is not yet fully understood.

Host-Pathogen Interactions in the Respiratory Tract

The specific interactions between *Mycoplasma gallisepticum* and the host cell are mediated by not only its primary adhesins mentioned previously, but the receptors of the host cell. Sialoglycoconjugates serve as receptors for an array of *Mycoplasmas* including *M. pneumoniae, M. genitalium, M. synoviae* and *M. gallisepticum* [11]. Specifically, *M. gallisepticum* can utilize these sialic acid moieties to bind human erythrocytes and other eukaryotic cells *in vitro* [186,43]. This pathogen, as discussed previously, can bind components of the extracellular matrix such as heparin, collagen type IV, fibronectin, and plasminogen [188,189,176].

Cytadherance is crucial in the respiratory epithelium and navigation of the mucociliary elevator is required for persistence. Other *Mycoplasmas,* such as *M. hyopneumoniae*, colonize the cilia of the trachea and bronchi by adherence to glycolipids in the pig epithelium [190]. This binding induces ciliostasis or stalling of the ciliary movement in the respiratory epithelium, allowing *M. hyopneumoniae* to traffic to the ciliary base [191]. *M. pneumoniae* exhibits a similar behavior of ciliary adherence utilizing gliding motility [192].

Ciliostasis has also been documented in *M. gallisepticum* infection of the avian respiratory tract. *M. gallisepticum* strain S6 and J1 induces ciliostasis in chicken tracheal organ cultures [193,194,195]. Chicken embryos infected with *M. gallisepticum* strain S6 also exhibit deciliation and erosion of the respiratory epithelial surface as early as 5 days post-infection, and this same effect was seen as early as 6 hours post-

infection when embryonic tracheas were infected *ex vivo* [196]. This phenomenon has also been described in turkeys infected with *M. gallisepticum*, which displayed lymphocytic infiltration in the nasal submucosa and ciliary loss in the sinuses [197].

Innate Immune Response

Upon infection with *Mycoplasma gallisepticum*, the avian host employs pattern recognition receptors (PRR) to recognize the invading pathogen. Chickens, the primary host of interest for *M. gallisepticum* in this dissertation, have a suite of pattern recognition receptors in the toll-like receptor (TLR) family that differ from their mammalian counterparts.

One example is TLR15, which is unique to avian and reptile organisms [198]. TLR15 was originally characterized as a sensor of yeast-derived compounds inducing the activation of NFκB signaling and subsequent IL-1β production [198]. Further study revealed that the expression of TLR15 was upregulated in response to bacterial infection [199,200,201] and may be directly induced by binding proteases during infection [202].

Another TLR of interest is TLR21, which is functionally homologous to human TLR9 [203]. This TLR recognizes CpG oligodeoxynucleotides, which are single-stranded DNA molecules that are present in microbial genomes and serve as pathogen-associated molecular patterns (PAMPs) to be recognized by PRRs during infection [203]. In contrast, TLR4 exists in both mammals and chickens but with only 46% identity

common between them [204]. TLR4 is able to sense bacterial lipopolysaccharide (LPS) during infection when associated with myeloid differentiation protein-2 (MD-2) [205].

TLR2 is also not unique to chickens and is associated with the sensing of peptidoglycan and lipoproteins in association with TLRs 1 and 6, and polymorphisms of TLR2 exist among various breeds of chickens who differ in their susceptibility to bacterial infection [206]. Heterophils, the polymorphonuclear cell of avian species similar to mammalian neutrophils, express TLR1/6/10, TLR2 types 1 and 2, TLR3, TLR4, TLR5, and TLR7 in chickens [207].

Lipoproteins of the virulent *Mycoplasma gallisepticum* strain R_{low} are recognized by chicken TLR2 in cultured, primary tracheal epithelial cells leading to the increased expression of pro-inflammatory genes related to NFκB signaling such as IL-1β, IL-6, IL-8, IL-12p40, CCL20, NOS-2 [208]. These effects are mirrored in DF-1 chicken embryo fibroblasts, in which both TLR2 and TLR6 upregulated in response to *M. gallisepticum* infection as well as downstream inflammatory genes such as MyD88, NFκB, IL-2, IL-6, and TNFα [209]. In chickens, *M. gallisepticum* infection induced increased expression of genes such as lymphotactin, CXCL13, CXCL14, RANTES, IL-6, and MIP-1β [210].

Recently, next-generation sequencing has allowed for a global, transcriptomic view of the tracheal immune response of chickens to infection with virulent *M. gallisepticum* R_{low} over time. Within the first 7 days after initial infection, a multitude of immune signaling pathways were found to be upregulated in response to *M. gallisepticum* including, but not limited to, TLR signaling, mitogen activated protein kinase (MAPK) signaling, Jak-STAT signaling, and nucleotide oligomerization domain (NOD)-like receptor signaling pathways [211]. The peak amount of differential gene

expression, both increased and decreased expression, occurred 3 days post-infection [211]. Interestingly, TLR4 and TLR15 were the most abundantly expressed TLRs in chickens across this 7-day time course of infection [211]. Metabolic pathways in the chicken host were also significantly activated 1- and 3-days post-infection, likely due to cellular stress in response to infection with virulent *M. gallisepticum* [211]. This cellular stress also relates to the abundant expression of two matrix metalloproteinases (MMPs) early in infection, MMP7 and MMP9, which are involved in extracellular matrix and tissue remodeling [211].

Two attenuated variants of *M. gallisepticum* discussed earlier in this dissertation, GT5 and Mg7, induced metabolic and immune-related genes to a much lower degree than virulent R_{low} in chickens during the first two days of infection [172]. The increases in TLR4 and TLR15 were not recapitulated in the attenuated *M. gallisepticum* infected chickens 1-day post-infection [172]. However, 2 days post-infection, the metabolic mutant Mg7 induced an increase in TLR4 and TLR15 expression above that of GT5, but not to the extent of virulent R_{low} [172]. Interestingly, TLR21 expression was significantly increased in R_{low} and GT5 infected chickens and decreased in Mg7 infected chickens both 1- and 2-days post-infection [Beaudet *et al*., 2019]. Induction of genes related to the Nlrp3 inflammasome in response to cell stress were also induced in response to infection with R_{low} or Mg7, such as caspase 1 (CASP1) and IL-1β [172].

In another one of its primary avian hosts, the house finch, virulent *M. gallisepticum* isolates Virginia 1994 (VA94) and North Carolina 2006 (NC06) induced the expression of pro-inflammatory cytokines in the nictitating membrane and Harderian gland [212]. Isolate NC06 was more virulent in house finches, and thus triggered a

stronger inflammatory immune response 3- to 6-days post-infection [212]. Notably, the expression of IL-1β strongly correlated with the load of *M. gallisepticum* in the conjunctival tissue of infected house finches [212].

Another growing area of research into the host response to *M. gallisepticum* infection is the induction of microRNAs (miRNAs). These miRNAs are differentially expressed during *M. gallisepticum* infection [213]. For instance, gga-miR-16-5p has been shown to promote apoptosis in DF-1 cells infected with *M. gallisepticum* which inhibits PI3K/Akt/NFκB signaling pathways [214]. In contrast, another miRNA, miR-130b-2p activates this same pathway in response to *M. gallisepticum* infection *in vitro* [215]. This differential effect on miRNA expression during infection may elude to mechanisms by which *M. gallisepticum* dysregulates the host immune response.

The cell-mediated innate immune response to virulent *M. gallisepticum* R_{low} challenge is characterized by an abundance of aggregates of B cells, CD4+ and CD8+ lymphocytes infiltrating the lamina propria of the trachea of infected chickens with expansion of lymphoplasmacytic and histiocytic infiltrates expanding in the tissue [216]. Chickens vaccinated with *M. gallisepticum* GT5 and subsequently challenged with virulent R_{low} yield lower numbers of these lymphocytes in the lamina propria of the trachea but have a larger population of IgA and IgG-secreting plasma and B cells in the trachea as early as 4 days post-challenge [216]. These same cells infiltrate not only the tracheal mucosa, but the air sacs of *M. gallisepticum* infected chickens and turkeys [217]. The pathologic manifestation of these responses results in the release of mucous granules in the respiratory epithelium and exfoliation of both ciliated and non-ciliated cells [218]. These epithelial cells lyse, and their cellular debris intermix with mucus to

form an exudate within the tracheal lumen and air sac [218]. Cells in the tracheal lumen are hypertrophic and exhibit a loss of ciliated cells compounded by increased mucosal thickness due to cellular infiltrates and edema in response to infection [218].

Section 2 – Broad Introduction to Influenza Viruses

History and Nomenclature of Influenza Viruses

The term “influenza” was originally used as a general descriptor for disease for a majority of history [219]. Until 1918, Pfeiffer’s bacillus, now known as the bacterium *Haemophilus influenzae*, was thought to be the cause of influenza before a filterable agent was isolated from influenza patient sputum as the true etiologic agent [220]. This filterable agent was used to reproduce disease in humans and ferrets, and thus described as the influenza virus [220].

Influenza viruses were named as “mucin-reacting” viruses, or myxoviruses [221]. This naming was then adjusted to orthomyxovirus to differentiate them from paramyxoviruses by differential structures [221]. Within this category, influenza viruses were named in alphabetical order by the chronological order of their isolation (i.e. Influenza A, B, C or D) [221].

Influenza A viruses can infect a range of hosts such as humans, pigs, horses, dogs, marine mammals and birds [222]. This particular subtype of influenza virus will be described in greater detail in the subsequent sections of this dissertation.

Influenza B viruses, first identified in 1940, primarily infect humans but can also cause disease in seals, horses, dogs, and pigs [223,224,225]. This subtype of influenza virus can cause more severe disease in immunocompromised humans and may be

responsible for annual epidemics, but not pandemics as with influenza A viruses [222,224].

Similarly, influenza C viruses, isolated in 1947, can infect humans and induces mild respiratory disease [226,227]. These viruses broadly circulate in areas across the globe, primarily in children [224]. Influenza C viruses can also be isolated from pigs, dogs, and camels with documented transmission between humans and pigs [224,228]. Most recently, influenza D viruses were identified in 2014 in swine, cattle and sheep with cattle being the natural reservoir [224,229]. These viruses share a 50% homology with influenza C viruses [224].

Influenza A Virus Characteristics

All influenza viruses are characterized by a segmented, negative-sense single stranded RNA (ssRNA) genome which replicates via RNA-dependant RNA polymerase activity [230,231]. The influenza A virus genome is contained in 8 RNA segments, encoding between 10 and 15 viral proteins depending on the specific isolate [232]. The influenza virion is spherical and particles range from 80 to 120 nanometers in diameter [222]. The viral particle is surrounded by a lipid bilayer containing approximately 500 protein spike projections per virion [222].

These protein spikes consist of the transmembrane hemagglutinin (HA), comprising 80% of the protein spikes, and the integral membrane protein neuraminidase (NA) which makes up the remaining 20% [222]. To date, there are 18 characterized subtypes of HA and 11 subtypes of NA in influenza A viruses [233].

Subtypes of influenza virus are named by their antigenic type (A, B, C, or D), host of origin, geographical origin, strain number, year of isolation, and combination of HA (H) and NA (N) subtype [234]. Famous influenza A pandemics include the Spanish flu of 1918 (H1N1), Asian flu of 1957-1958 (H2N2), Hong Kong flu of 1968-1969 (H3N2), and the Swine flu of 2009 (H1N1) [222].

Both HA and NA are crucial components of influenza virulence. Hemagglutinin (HA) binds to host ligands containing *N*-acetylneuraminic acid, or sialic acids, and this binding specificity determines tissue and species tropism of the virus [34,235]. For instance, influenza A viruses of human hosts preferentially bind the terminal α(2,6)-linked sialic acids abundant in the human upper respiratory epithelium and viruses of avian hosts bind the α(2,3)-linked sialic acids of the avian respiratory and gastrointestinal epithelium [34]. Porcines, however, can become infected with both mammalian and avian strains of influenza A virus due to the abundance of both α(2,3)- and (2,6)-linked sialic acids in their tracheae [34]. The implications of two cohabitating influenza A viruses within a single host will be discussed in a later section of this dissertation.

Viral Replication, Assembly, and Release

The HA protein is the mediator of fusion of influenza virus to the host cell membrane. HA binds sialylated receptors on the host cell and is engulfed via endocytosis into the host cell [236]. The virus then forms an endocytic vesicle which will fuse with the endosomal compartment and release the virus into the endosome

[237,238]. Once within the endosome, the virus responds to the low pH environment by a conformational change in the structure of HA to expose the fusion peptides in its trimeric stem region [239]. This allows for a membrane fusion between the viral lipid bilayer and the host endosome, as well as opening of the M2 ion channel [240].

Within the endosome, the virus will un-coat and export viral ribonuclear protein (RNP) comprised of viral RNA, RNA-polymerase, and viral nucleoprotein (NP) [241,242]. Viral RNA is trafficked to the host cell nucleus and new viral genome is synthesized there utilizing host pre-mRNA caps before export [243,244]. The freshly transcribed viral RNAs are transported into the host cell cytoplasm for translation on host ribosomes [245]. Progeny virions are finally assembled at the membrane and released by budding from the host cell mediated by NA and the matrix protein, M1 [245,246,247,147].

There are an array of proteins encoded by influenza A virus that contribute to various stages of the viral life cycle. Polymerase basic protein 2 (PB2), is involved in the transcription of viral genomic RNA [249]. Polymerase basic protein 1 (PB1) interact with polymerase acidic protein (PA) in the elongation of viral RNA [250]. PB1-F2 targets host mitochondria and trigger apoptosis [251]. PA also works to bind PB1 in the assembly of viral RNPs alongside NP during replication [252,253].

The matrix protein (M1) is involved the assembly of progeny virions by binding to viral RNP to associate with non-structural protein 2 (NS2), the viral nuclear export signal [254,255]. Non-structural protein 1 (NS1), however has many functions including anti-interferon activity and the regulation of viral RNA nuclear export [254,255]. The ion channel protein, M2, mediates the uncoating of the viral capsid and controls the pH of

the host Golgi apparatus to prevent a premature change in HA confirmation [240,256,257,258]. Lastly, influenza NA, as mentioned earlier with bacterial sialidases, cleaves host sialic acids during viral maturation and budding from infected host cells [259]. NA is a popular target for anti-influenza drug development and may work in concert with bacterial NAs or sialidases during infection, as will be discussed later in this dissertation.

Antigenic Shift and Drift

When a singular host cell is infected with multiple different influenza viruses, a phenomenon known as antigenic shift can occur. Within the host cell, there is reassortment of the segments of viral RNA which will then swap parts of the genome between viruses when they assemble and bud from the host [260]. This reassortment can lead to a broadening of tissue or host tropism and can be the start of an influenza pandemic [224].

The most common hosts for these antigenic shifts are pigs, quails and bats due to the abundance of both α(2,3)- and (2,6)-linked sialic acids to accommodate mammalian and avian influenza A viruses [224]. Antigenic drift, however, is the gradual accumulation of viral RNA mutations over time due to the error prone nature of influenza RNA-dependent RNA polymerase [261]. These mutations lead to more subtle changes in the virus which can be responsible for seasonal variations in the circulating influenza A strains [261].

Antiviral Drugs and Vaccines

There are two main classes of anti-influenza dugs; M2 ion channel inhibitors and neuraminidase inhibitors. M2 inhibitors are effective in both preventing infection with influenza A virus and reducing the duration of infection by inhibiting viral replication [262,263]. Examples of M2 inhibitors include amantadine (Symmetrel) and rimantadine (Flumadine) [262]. Although initially effective, M2 inhibitors are no longer a recommended course of treatment because of emerging resistance to the available inhibitors [263].

The other class of anti-influenza drugs is neuraminidase (NA) inhibitors. One such inhibitor is zanamivir (Relenza), which is an altered form of another NA inhibitor 2-deoxy-2,3-dehydro-N-acetyl-neuraminic acid (DANA) to enhance specificity for viral NA over host NA [34]. This drug has poor oral bioavailability and must be inhaled for efficacy [264,265]. Perhaps the most popular NA inhibitor is oseltamivir (Tamiflu). Oseltamivir is administered orally and is commonly stockpiled in anticipation of avian influenza pandemics [265]. This drug was designed for competitive inhibition of the active site of the viral NA [264,265]. The pro-drug form, oseltamivir phosphate, is prescribed to patients and when ingested, undergoes an ester hydrolysis reaction in the patient's liver to its active form, oseltamivir carboxylate [266].

Neuraminidase inhibitors, such as oseltamivir and zanamivir, have been effective in shortening the length of illness and viral shedding in human patients if given within the first 24 to 48 hours of symptom onset [267]. Both zanamivir and oseltamivir are available worldwide for human use [277]. Similar drugs, such as laninamivir (Inavir) are

available in Japan and peramivir (Rapiacta or Permaflu) are available in China, Japan, South Korea and the United States [268]. Oseltamivir and zanamivir can be prescribed as prophylactic measures, however this is not recommended for peramivir and laninamivir [267].

This prophylactic use, however, can lead to the development of neuraminidase inhibitor resistance in circulating influenza strains after prolonged use of sub-therapeutic concentrations of drug [267]. Emerging resistance to these neuraminidase inhibitors is a growing area of research to develop new drugs and drug targets. Recently, the World Health Organization (WHO) analyzed 13,581 neuraminidase gene sequences from public databases of influenza A virus strains from the 2016-2017 influenza season [267]. Of the influenza viruses tested, 94% were sampled from the Western Pacific region, North and South America, and Europe, whereas the remaining portion represented the Eastern Mediterranean region, Africa, and South East Asian region [269]. Only 0.5% of the neuraminidase sequences from these viruses displayed likely neuraminidase inhibitor resistance, or reduced inhibition of activity, however this proportion may grow over time and does not encompass the global influenza distribution.

Vaccination for human influenza is a major public health endeavor, headlined by the WHO. The FLUVACS clinical trial analyzed influenza vaccination strategies during the 2007-2008 influenza season [270]. The live-attenuated nasal influenza vaccine, FluMist, is efficacious but this does not correlate with an increase in HA or NA inhibition titers in human patients [270]. The inactivated vaccine, Fluzone, however does correlate increased efficacy with NA inhibition titers in vaccinated patients [270]. The most widely

used influenza vaccine is an inactivated, trivalent vaccine administered by injection [270]. Another type of influenza vaccine, FluBlok made by Protein Sciences, is a recombinant HA vaccine marketed to patients with an egg allergy, as its development does not merit the use of embryonated chicken eggs with an up to 40% reduction in influenza infection in elderly recipients [271]. These vaccines are carefully designed to protect against the forecasted influenza strains of relevance for a given season, which lasts 7 to 8 months [271].

Vaccination efforts also exist for cases of animal influenza and will be discussed in more detail in subsequent sections of this dissertation. The World Organization for Animal Health (OIE) and Food and Agriculture Organization of the United Nations (FAO) collaborated to form a team of experts on animal influenza (OFFLU) as a means to advise veterinary, scientific, and agricultural professionals on risk and management of animal influenza infections [272]. In 2009, the U.S. Department of Agriculture (USDA) formed a national swine influenza A virus surveillance system which has paved the way for similar systems in European nations [273,274].

Sub-Section 2-1: Avian Influenza Viruses

Pathobiology

As mentioned earlier, a noteworthy group of hosts of influenza A virus are avian species. Of the possible 16 HA and 9 NA subtypes of influenza a virus, most have been found in waterfowl which serve as a common reservoir host for influenza [224]. These strains of avian influenza A virus (AIV) preferentially bind α(2,3)-linked sialic acid moieties present in the respiratory and gastrointestinal tract of galliform birds and waterfowl [275]. Chickens, quails, partridges, turkeys, pheasants, ostriches, and mallard ducks express both α(2,3)- and α(2,6)-linked sialic acids differentially in abundance within their nasal cavities, tracheae, and lungs which contributes to the varied host and tissue tropism of different AIV strains [276].

AIVs are categorized into two broad types based on their pathogenicity in avian species; highly-pathogenic AIV (HPAIV) and low-pathogenic AIV (LPAIV). HPAIV has been referred to as “fowl plague” throughout history before AIV was better understood [277]. HPAIV primarily replicates in the avian respiratory tract and sheds readily from the trachea [275]. This infection can spread readily to other internal organs such as muscle, intestinal tract and central nervous system causing severe mortality [275].

HPAIV strains are typically limited to the H5 and H7 HA subtypes of influenza A virus [278]. Notably, HPAIV strains that cause severe mortality in gallinaceous birds often do not do the same in waterfowl and wild birds and vice versa [278]. These HPAIVs are thought to have evolved from LPAIVs as they persist in a wild bird population over time [278].

Gallinaceous birds infected with high mortality strains of HPAIV may exhibit central nervous system lesions such as multifocal gliosis, neuronal degradation and lymphocytic meningitis [279]. These lesions may be accompanied by heart lesions such as diffuse lymphocytic perivascular cuffing, vacuolation of myocardial cells, as well as general histiocytic infiltration, coagulative necrosis of the thymus, kidney and bursa, fibrosis, catarrhal tracheitis, and pneumonia [279].

The majority of AIVs belong to the LPAIV category [280]. LPAIV replicates in the intestinal tract of infected birds and is this shed from the cloaca for fecal-oral transmission in a waterfowl [275]. These viruses, in contrast to HPAIVs, are limited to the epithelial tissues in infected hosts and due to the subversive nature of disease, transmission to poultry hosts may easily go unnoticed [275]. Lesions in LPAIV H5N2 (A/chicken/Pennsylvania/21525/83) and H4N8 (A/chicken/Alabama/7395/75) infected chickens may present as a diffuse epithelial hyperplasia in the trachea with lymphocytic infiltrates in the tracheal submucosa [279]. This may present clinically as general upper respiratory signs, swelling of the head, and lethargy [280]. More severe cases may yield diffuse lymphocytic pneumonia, necrosis of the spleen, bursa, thymus and kidneys, and enteritis [279]. In waterfowl, the airway becomes infected with LPAIV when the birds exhibit dabbling behavior in the water [275]. This results in a mild tracheitis and pneumonia 2 days post-infection but is undetectable in the airway as soon as 3 to 4 days post-infection [275].

Certain LPAIVs, such as H9N2, are endemic in poultry populations in the Middle East, North Africa, and Asia after circulating from China in 1994 [280]. The significance of such LPAIV outbreaks will be discussed further in later sections of this dissertation.

However, most LPAIV infections often goes unnoticed due to the mild and often subclinical nature of disease in most avian hosts [281]. In the field, LPAIV can be suspected when populations of birds show a decrease in water and feed consumption and minimal movement toward food or water [281]. Infected birds typically recover from LPAIV infection in the respiratory tract if they do not have compounding health issues, however effects on egg production can be severe in chickens and turkeys making the disease of concern to agricultural professionals [281].

Significance and Control Measures

AIV is a pathogen of importance not only for animal health, but human health and industrial practices as well. It has been estimated that a pandemic of AIV would initiate an economic loss of between $100-200 billion dollars in the United States alone in 2004 currency values [282]. Asian nations experienced outbreaks of HPAIV in 2003 and 2004 which resulted in the direct loss of 44 million birds, accounting for around 17.5% of the total poultry population in Vietnam [282]. This loss accounts for as much as 1.8% of the gross domestic product of the entire nation, equating to approximately $76-450 million United States dollars in 2004 currency values [281]. Such a pandemic would be crippling in nations such as the United States or Brazil who account for almost 70% of the global supply in the poultry trade [282].

These economic concerns are not limited to HPAIV isolates. For example, LPAIV strain H9N2 was identified as the culprit of an outbreak in China between 1992 and 1994, leading to drastic economic losses resulting from losses in egg production and

poultry mortality [283]. LPAIV H9N2 was also responsible for a field outbreak in Korea in 1996 which lead to efforts to stamp out the disease in infected chickens [283].

Control measures for AIV are crucial in preventing outbreaks of disease in wild and agricultural populations of birds. Although LPAIV does not carry the same stigma of rapid mortality and severe disease as HPAIV, surveillance for this often subclinical, underestimated pathogen is still of great importance [275]. LPAIVs of the H7 subtype in particular can be readily transmitted between chickens and turkeys, so field surveillance measures are critical in controlling spread between wild and domestic birds [284].

Vaccination efforts against AIV vary across the globe. Surprisingly, many countries do not vaccinate against HPAIV. In the 1990s, some prophylactic use of inactivated AIV vaccines were employed in Mexico and Pakistan to control outbreaks of both HPAIV and LPAIV [272]. In the years following, inactivated virus vaccines have been the recommended course of action over live-attenuated vaccines, regardless of the subtype per OIE standards [272,285]. Overall, AIV vaccines are comprised of inactivated virus in oil-emulsion, and only a minority of AIV vaccines remain live, viral vectored vaccines [286]. In nations such as Egypt, Vietnam, China, Mexico, and Indonesia, vaccination is targeted against H5, H7, and H9 AIVs to combat endemic AIVs in those regions [287]. These vaccination protocols have also been useful in the control of HPAIV to prevent nations from culling, or stamping out, populations of wild and domestic birds to protect the food supply [281].

One concern, however, is that vaccination may prevent the manifestation of clinical signs, but not infection, with HPAIV and may unintentionally contribute to the spread of the virus to endemic status in a given region [288]. Targeted vaccine

strategies for HPAIV in specific geographic locations and host species is rare, accounting for less than 1% of all AIV vaccinations [286]. Vaccination strategies for AIV are primarily focused on poultry, however limited vaccinations effots have been employed on captive birds such as zoo animals [286]. Wild birds, although often a resevior for AIV, are not a target of vaccination efforts due to logistical concerns [286].

Vaccination against LPAIV is a growing effort due to the probability of the mutation of a subclinical infection of LPAIV to HPAIV when circulating in the field [281]. In the 1970s, inactivated virus vaccines against H5 and H7 LPAIV strains have been used in turkeys, as well as preventative vaccination against H5N1 AIV in outdoor poultry and zoo birds in Europe [272].

Even in regions where vaccination is not supported, some poultry farmers will expose pullets, or young chickens, to AIV to prevent a later outbreak that would cause severe losses in production due to disease or eradication measures [289]. Evidence also suggests that oseltamivir, the NA inhibitor used to treat human influenza infections, is effective in reducing the load of H9N2 and H6N2 LPAIV in the tracheae and cecal tonsils of infected chickens and ducks [291]. Prevention of LPAIV infection is also crucial in that, when compounded by a secondary infection, disease severity can be dramatically enhanced. These co-infection scenarios will be discussed in greater detail in later sections of this dissertation.

Host Innate Immune Response

Upon infection with influenza virus, the host can sense and respond to this invasion with an assortment of signaling cascades. For instance, innate sensors of influenza virus infection include TLR3, TLR7, RIG-I, NLRs, and NLRP3 [292]. An associated signaling molecule, MyD88, is a critical member of the signaling cascade to recruit innate immune cells and to coordinate cytokine and chemokine production upon viral infection [292]. One such class of innate immune cells are natural killer (NK) cells, activated by TLR7 during influenza infection [293].

Although the host is equipped with an array of pattern recognition receptors to respond to infection, influenza virus has adapted with mechanisms of immune evasion and modulation. One such mechanism is the induction of suppressor of cytokine signaling (SOCS) by influenza, which in turn inhibits TLR and antiviral IFN signaling [294]. The viral protein NS1 is another facet of influenza immune evasion. One innate response to influenza infection is signaling through the retinoic acid-inducible gene I (RIG-I). This response also involves melanoma differentiation-associated gene 5 (MDA5), interferon-beta promoter stimulator 1 (IPS-1), and interferon-regulated factor 4 (IRF3), all contributing to an anti-influenza IFN-β response [295]. The NS1 protein of influenza virus can negate this response by repressing RIG-I signaling and evading the subsequent IFN-β activity [295].

Influenza infection can also trigger cell death in the host in a variety of ways. Infection of a host cell with influenza can induce the expression of Fas and Fas-ligand on the cell surface, which then triggers programmed cell death via apoptosis [296]. Apoptosis can also be induced during infection by the viral dysregulation of the host type 1 interferon response [297]. In a human host, this dysregulation induces pulmonary

injury from alveolar macrophages expressing IFN-β, thus contributing to alveolar epithelial damage [297]. This response triggers expression of TNF-related apoptosis-inducing ligand (TRAIL), contributing to cell death in the host respiratory tract and a subsequent manifestation of pneumonia [297]. Apoptosis can also be directly induced by the influenza proteins PB1-F2 which target the host mitochondria and induce pore formation [298].

Another mechanism of cell death induction in response to influenza infection is the activation of the host inflammasome. To accomplish this, the host must recognize the virus by activation of PRRs which will then signal with other members of the inflammasome complex, such as NLRP and its adaptor Apoptosis-Associated Speck-Like Protein Containing CARD (ASC) [298]. This adaptor then activates the maturation of caspase-1 which cleaves pro-IL-1β and pro-IL-18 cytokines for secretion and inflammatory action [298]. This caspase-1 activity is a facet of pyroptosis, or cell death by inflammation [298]. The inflammatory cytokines IL-1β and IL-18 are also critical in activation of the inflammasome [298]. This activation of ASC and caspase-1 signaling serves as a primer for later CD8+ T cell responses to immunodominant influenza antigens, and the downstream production of IgA by B cells [298].

The influenza porin protein M2 can also trigger NLRP3 activation of the inflammasome by modulating the intracellular ion concentrations within the host cells [298]. The NLRP3 inflammasome activation induces a TH17 and TH1 response in the host, which promotes CD4+ T cell and memory T cell responses [298].

In the human respiratory tract, the induction of pro-inflammatory IL-6 and IFN-α increases up to 2 days post-infection, and this increase correlates with climbing

influenza titers, increases in body temperature, mucous production, and clinical symptom severity [299]. IL-6 is also abundant in the circulatory system at this time [299]. At 3 to 4 days post-infection, TNF-α peaks in concentration in both the upper respiratory tract and serum, followed by IL-8 four to six days post-infection both of which correlating with decreasing viral loads and respiratory symptoms of infection [299]. In humans with autosomal deficiencies in TLR3, the dysfunctional TLR3-dependent type I and type III interferon response contributes to influenza virus infection progression to pneumonitis [300].

The innate immune response to AIV is somewhat unique, and the response in avian hosts yield even further intricacies to the innate response to influenza. For instance, in contrast to LPAIV, HPAIV has tropism for endothelial cells in poultry due to its trypsin-independent HA cleavage during replication [301]. Within the possible avian hosts, the response to influenza infection varies even further. In ducks, infection with HPAIV induces a more robust innate immune response overall compared to LPAIV, and this response can produce inflammation in the brain [302]. HPAIV infection induces the inhibition of the anti-inflammatory mediator STAT-3 by HPAIV H5N1 in chickens [310,311]. This induces a unique pro-inflammatory cytokine storm in the chicken host, which is not replicated in ducks during the same infection [310,311].

Although LPAIV typically replicates slower than HPAIV, it does induce a more significant IFN-β and interferon stimulating gene (ISG) response [230]. These LPAIVs, such as H9N2, also stimulate a weak IFN-α response but still contribute to increased expression of interleukins, TLR activation, and RIG-I, Jak-STAT, and NF-κB signaling

[294]. HPAIVs, on the other hand, induce a drastic type 1 interferon response in the lung during infection of an avian host [294].

In contrast to ducks, chickens do not possess RIG-I which contributes to a differential susceptibility to HPAIV infection between ducks and chickens [275,294]. During LPAIV infection in mallards, RIG-I and myxovirus resistance gene (Mx) are upregulated initially, then returning to a baseline level by 2 days post-infection [304]. In place of RIG-I, chickens utilize MDA5 in its place to signal the same type 1 IFN response via MAVS, IRF3 and IRF7 [294].

Another unique aspect of the avian response to AIV is the downstream signaling of TLR3. In mammalian hosts, TLR3 recognizes dsRNA for the later activation of NFκB and type 1 interferons [305]. In geese, TLR3 is upregulated in the lungs and brain during AIV H5N1 infection alongside IL-6 and IFN-γ [305]. This type 1 interferon response in mammals is mediated by IRF3, which is absent in chickens who instead utilize IRF1 to regulate an IFN-β antiviral response [306]. TLR3 is also protective against LPAIV H4N6 infection in chicken eggs [307].

Other relevant TLRs in AIV include TLR21, which is upregulated in response to LPAIV H9N2 infection in geese [308]. In chickens pro-exposed to LPAIV H9N2 followed by HPAIV H5N1, several TLRs are differentially expressed. TLR1, TLR2, TLR5 and TLR7 are decreased in expression after the secondary infection with HPAIV in contrast to TLR3 and TLR15 which are increased in expression [309]. Other genes of interest include the downregulation of IFN-α, IFN-β, IFN-γ, IL-1β, IL-2, IL-4, IL-8 and TGF-β and the upregulation of TNF-α [309]. Overall, the innate immune response to AIV is a

nuanced process made further complex by the viral evasion and modulation of the host response.

Sub-Section 2-2: Influenza A Virus H3N8

Mammalian H3N8

The influenza A virus subtype H3N8 has been recently implicated in disease in an array of mammalian species. In 2015, there was an outbreak of H3N8 equine influenza in Malaysia [312]. Infected horses treated with oseltamivir phosphate had reduced viral shedding and fever with an additional reduction in the likelihood of a secondary bacterial pneumonia from *Streptococcus equi* subsp. *zooepidemicus* infection [312]. It is hypothesized that this equine H3N8 was transmitted to canines, resulting in the current H3N8 canine influenza with associated respiratory disease [313,314].

This proposed introduction occurred in 2004, when H3N8 was first isolated in greyhounds in the United States exhibiting respiratory disease [315]. Once established in the canine host, H3N8 is easily horizontally transmitted between dogs in a closed environment such as a shelter [316]. This canine H3N8 has experienced robust host-adaptation and cannot be transmitted between dogs and avian species such as chickens, turkeys, or ducks [317]. The respiratory disease caused by canine H3N8 is variable in severity, however co-infection in dogs with other respiratory pathogens such as *S. equi* subsp. *zooepidemicus* can be common and vaccination against canine H3N8 has been effective in reducing disease in co-infected dogs [318].

In 2011, an outbreak of H3N8 was detected in 162 New England harbor seals with drastic mortality from pneumonia [320]. Unlike canine H3N8, the H3N8 isolated

from New England harbor seals retains the ability to bind α(2,3)-linked sialic acids similarly to AIV which could indicate possibility for cross-species transmission [321].

Avian H3N8

In avian hosts, influenza A virus H3N8 is classified as a low pathogenic avian influenza virus (LPAIV). This virus has been detected in areas across the globe in an array of hosts. For instance, H3N8 was detected in wild birds on the central Peruvian coast from 2006 to 2007 [322]. H3N8 and H4N6 are the most prevalent AIV strains isolated in mallards in northwestern Minnesota in the United States between the years of 2007 and 2016 [323].

In China, H3N8 accounts for 25% of AIV detected in domestic ducks and wild birds among other hemagglutinin subtype 3 viruses and has the ability to cross into chicken hosts [324]. H3N8 also co-circulates with H3N2 and a newly emerged H3N6 in Chinese wild and domestic birds [325]. In Europe, oropharyngeal and cloacal H3N8 shedding is limited in passerine birds such as house sparrows and European starlings [326].

H3N8 (A/Mallard/MN/199106/99) does not produce gross lesions in experimentally infected mallards, however it does induce lymphocytic tracheitis and laryngitis early in infection and can be shed up to four days after infection [326]. It has also been documented that H3N8 infection can result in the decrease of α(2,3)-linked sialic acids in the bursa of Fabricius and enterocytes of experimentally infected ducks [326]. In young mallards, H3N8 is shed more abundantly than in older ducks [327].

Ducks that were previously infected with H3N8 are later protected from infection with closely related strains of AIV [328,329,330].

Section 3 – Viral and Bacterial Co-Infections of the Respiratory Tract

Mammalian Respiratory Co-Infections

Concurrent infection with viral and bacterial pathogens in the respiratory tract are a common phenomenon among humans and other mammals. These co-infections, also referred to as superinfections, thrive in a compromised host and often lead to drastic increases in disease severity and mortality. The underlying mechanisms of these co-pathogen relationships include dysfunction of the lung and airway physiology, changes in viral tropism, increased availability of receptors for pathogen attachment, immunomodulation of the host, and increases in inflammatory signaling [331].

As mentioned earlier in this dissertation, Mycoplasmas infect a wide variety of mammalian hosts and these infections can be compounded by viral co-infection. *Mycoplasma hyorhinis* has been documented as a co-pathogen of porcine reproductive and respiratory syndrome virus (PRRSV) in pigs, both contributing to more severe lung lesions and the development of the porcine respiratory disease complex (PRDC) [332].

A similar dynamic exists between PRRSV and *Mycoplasma hyopneumoniae* within the PRDC. Pigs co-infected with *M. hyopneumoniae* and PRRSV are protected from exacerbation of disease from co-infection when given a vaccine against *M. hyopneumoniae* [333,334,335]. *M. hyopneumoniae* and PRRSV are also co-pathogens of porcine cirovirus type 2 (PCV2), the causative agent of postweaning multisystemic wasting syndrome in pigs [336]. Co-infection with *M. hyopneumoniae* and PCV2 enhances viral replication *in vitro* [337].

In humans, there have been a number of cases of co-infection with *Mycoplasma pneumoniae* and influenza virus. One such case was an elderly female patient in China co-infected with *M. pneumoniae* and AIV H7N9 [338]. In this patient, *M. pneumoniae* persisted to a more chronic state of 33 days after initial treatment, whereas H7N9 was no longer detected after NA inhibitor treatment [338]. Another case related to a young adult female patient co-infected with *M. pneumoniae* and influenza B virus [339]. This patient experienced severe fever and respiratory signs for over 1 week after initial treatment [339]. A recent survey of 445 patients suffering from acute febrile respiratory syndrome in China demonstrated that co-infections with influenza virus and *M. pneumoniae* were among the most commonly detected in this population [340]. In these cases of influenza and *M. pneumoniae* co-infection, very little is understood about the precise mechanistic relationship between the two pathogens.

Influenza co-infections are among the most commonly studied in the respiratory tract among many hosts. For instance, canine influenza and *Staphylococcus pseudointermedus* co-infection often causes severe respiratory disease in canines [341]. A mouse model of this co-infection showed increases in both bacterial and viral load in co-infected animals over their mono-infected counterparts [341]. Co-infected mice also exhibited more severe histologic lesions in the brain, spleen, and lung as well as increased expression of IFN-γ, IL-6, TNF-α, and lymphotactin [341].

Another frequent co-pathogen of influenza is *Klebsiella oxytoca*, often associated with opportunistic infection of hospitalized patients during the influenza season [342]. In a mouse model, co-infection with these pathogens significantly increased weight loss, pulmonary inflammation, and mortality [342]. The pulmonary inflammatory response to

this co-infection was characterized by macrophage skewing to an inflammatory phenotype, however this robust response did not effectively reduce the pathogen load in co-infected mice [343].

Mice co-infected with influenza and methicillin-resistant *Staphylococcus aureus* (MRSA) rely on an effective pro-inflammatory response to clear infection. Influenza infection alone induces a strong type I interferon response which represses TH17 activation, and this signaling is controlled by the transcription factor STAT1. Mice lacking STAT1 are significantly more susceptible to airway inflammation following infection with influenza or MRSA but are protected against co-infection via reduced MRSA colonization and mortality [343]. In mice lacking STAT2, another transcription factor of type I interferons, a similar phenomenon exists during influenza and MRSA co-infection as well as increased accumulation of M1 and M2 macrophages [344].

Perhaps the most widely studied co-pathogen relationship is between influenza virus and *Strepcococcus pneumoniae*. These two pathogens each have an active neuraminidase, and these viral and bacterial neuraminidases work synergistically during infection [345]. When the neuraminidase inhibitor oseltamivir is used to disrupt viral neuraminidase activity, the survival of co-infected animals significantly increases [345]. During co-infection, influenza neuraminidase cleaves sialic acid moieties on the surface of host lung cells to expose *S. pneumoniae* receptors, and this enhanced colonization is the underlying basis for the development of secondary bacterial pneumonia [345]. The *S. pneumoniae* neuraminidase gene, *nanA*, enhances bacterial biofilm formation during co-infection with influenza virus [346].

Host sialic acids are the primary host-derived metabolite used by *S. pneumoniae* for growth and persistence in the host [347]. Infection with influenza increases the availability of these sialic acids via sialylated mucins and the desialylation of host glycoproteins [347]. *S. pneumoniae* mutants lacking the genes for sialic acid metabolism do not exhibit enhanced growth during co-infection with influenza virus compared to wild-type *S. pneumoniae* [347]. A sialidase, or neuraminidase, fusion protein can be utilized as a broad-spectrum influenza therapeutic with significant reduction in the development of secondary *S. pneumoniae* infection in mice co-infected with influenza [348]. Perhaps most notably, *S. pneumoniae* neuraminidase activity is able to rescue influenza replication in the presence of the viral neuraminidase inhibitors zanamivir and DANA *in vitro* [349].

Co-infection with *S. pneumoniae* and influenza also induces increased activation of TLR2, MyD88 and the NLRP3 inflammasome in mice [350]. Other key immune gene modulation includes the upregulation of TGF-β, fibronectin, and integrins during influenza infection of human epithelial cells which enhances *S. pneumoniae* attachment to host cells [351]. TLR4 signaling is also key in the development of respiratory pathology in rats infected with influenza [352]. Mice co-infected with influenza and *S. pneumoniae* exhibited more severe lung pathology and mortality over mono-infected mice, however this enhanced pathology was reversed when mice were treated with a TLR4 agonist between influenza and *S. pneumoniae* infections [352]. Treatment with this TLR4 agonist also reversed the suppression of IFN-β, CXCL1, and CXCL2 by influenza infection, allowing for proper neutrophil activation and pathogen clearance [352]. Another key inflammatory molecule is IL-17A, which reduces *S. pneumoniae*

colonization in mice but enhances respiratory inflammation when these mice are co-infected with influenza virus [353].

Avian Respiratory Co-Infections

Viral and bacterial co-infections of the respiratory tract are also of great relevance to avian species, and in light of the impact of AIV on avian populations these co-infections are even more worthy of understanding. LPAIV H9N2 enhances respiratory distress in birds co-infected with the foodborne pathogen *Bacillus cereus* [354]. H9N2 also induces move severe airsacculitus in SPF birds co-infected with bacterial pathogens such as *Chlamydia psittaci, Ornithobacterium rhinotracheale,* and the fungal pathogen *Aspergillus fumigatus* which are all pathogens found in the Chinese poultry industry [355].

Another prominent co-pathogen of influenza in avian species is *Eschericia coli*. Turkeys co-infected with LPAIV H6N1 and *E. coli* exhibit enhanced disease severity in terms of respiratory lesion development and mortality [356]. Similar impacts on pathogenesis are seen in chickens co-infected with LPAIV H3N8 and *E. coli* or LPAIV H9N2 and *E. coli* [357,358]. The order and timing of this co-infection impacts measures of disease severity differently, however all combinations exacerbate disease over mono-infected chickens [359]. Chickens infected first with LPAIV H9N2 followed by *E. coli* yielded increased lung lesions up to 3 days post-infection compared to mono-infection, with phagocytic infiltrates of the respiratory tract increasing with *E. coli* load [360]. Avian macrophages infected with LPAIV H9N2 followed by a stimulation with *E. coli*

lipopolysaccharide increased the expression of IL-6, IL-1β, CXCL1, CXCL2, TLR4, and MDA5 compared to macrophages infected with H9N2 alone [361].

Other relevant LPAIV co-pathogens include *Staphylococcus aureus*, *Haemophilus paragallinarum*, and *Ornithobacterium rhinotracheale*, all of which increase disease severity in chickens over mono-infected animals [362,363]. In wild birds, AIV infection is commonly associated with pathogens such as *Mycobacterium*, *Salmonella*, and *Mycoplasma* [364,365]. Vaccination of commercial layer hens with *M. synoviae* leads to an increased replication of LPAIV H9N2 [366]. Mycoplasmas, such as *M. synoviae* and *M. gallisepticum*, are also associated with co-infection with infectious bronchitis virus (IBV) and avian metapneumovirus in poultry flocks [367]. Vaccination of chickens with *M. gallisepticum* ts-11 and 6/85 also imparts some non-specific protection against IBV infection, and this vaccination significantly decreased ciliary damage during co-infection [368].

Co-Infection with *M. gallisepticum* and Low Pathogenic Avian Influenza Virus

Within the category of avian viral and bacterial co-infections, the synergistic pathogenesis of *Mycoplasma gallisepticum* and low pathogenic avian influenza virus (LPAIV) is the focus of this dissertation. In the literature, it has been established that infection with both *M. gallisepticum* and LPAIV exacerbates disease both *in vitro* and *in vivo*.

Stipkovits *et al*. demonstrated this co-infection relationship *in vivo* in two separate publications using *M. gallisepticum* strain 1226 and LPAIV H3N8

(A/mallard/Hungary/19616/07) [367,368]. Both publications used an aerosol challenge method to inoculate chickens with either *M. gallisepticum* 1226, LPAIV H3N8, or *M. gallisepticum* followed by H3N8 7 days later [369,370]. After 8 days of observation following the secondary infection, chickens were sacrificed in both published studies [369,370].

In one publication, Stipkovits *et al*. observed that clinical signs, such as respiratory rales and difficulty breathing, were more severe in co-infected chickens than mono-infected chickens [369]. These co-infected chickens also exhibited a reduction in weight gain compared to mono-infected chickens over the course of the experiment [369]. Finally, isolation of *M. gallisepticum* 1226 was enhanced in sampling from co-infected chickens in the respiratory and inner organs over *M. gallisepticum* mono-infected chickens in this experiment [369].

In a similar publication, Stipkotivs *et al*. instead focused more on the pathologic lesions induced by co-infection with *M. gallisepticum* and H3N8. Chickens co-infected with these pathogens exhibited more severe gross pathologic lesions, such as airsacculitis and peritonitis, when compared to chickens infected with either pathogen alone [370]. Macroscopic lesions in the trachea, left and right thoracic air sacs, and peritoneum were also enhanced in co-infected chickens over mono-infected chickens in these experiments [370]. These data corresponded to more severe clinical scoring of histopathologic lesions of tracheitis, bronchitis, and interstitial pneumonia in the co-infected chickens of this publication [370]. This pathology was characterized by increased tracheal and bronchial mucosal thickness as well as lymphocytic and

histiocytic infiltration accompanied by degeneration and metaplasia of the respiratory epithelium [370].

To examine the co-pathogenesis relationship *in vitro*, Sid *et al*. utilized both chicken and turkey tracheal organ cultures (TOCs) to model co-infection with *M. gallisepticum* and LPAIV H9N2 (A/chicken/Saudi Arabia/CP7/1998) [371]. These TOCs were first infected with *M. gallisepticum* followed by H9N2 either 24 or 72 hours after the primary infection [371]. After 48 or 96 hours following the primary infection, data were collected from all groupings of TOCs [371]. Isolation of *M. gallisepticum* was increased in co-infected chicken and turkey TOCs, and H9N2 isolation was differentially affected by co-infection across time points [371]. TOCs co-infected with both pathogens also exhibited a larger proportion of apoptotic cells per tracheal ring compared to mono-infected TOCs [371]. In turkey TOCs, IFN-α and IFN-β expression measured by qRT-PCR were increased over mono-infected TOCs [371]. IFN-γ expression, however, was suppressed in co-infected chicken TOCs when compared to mono-infection with H9N2 [371]. Most notably, co-infection with both pathogens significantly increased the degree of ciliostasis in both chicken and turkey TOCs when compared to mono-infected TOCs [371].

These scientific works illustrate potential contributing factors to *M. gallisepticum* and LPAIV co-pathogenesis. However, there are still openings in our understanding of the precise nature of the relationship between these pathogens and what mechanisms underly their co-pathogenesis in the natural, avian host. Both *M. gallisepticum* and LPAIV are of great relevance to the agricultural industry and merit further elucidation of their synergistic infection to treat or prevent this phenomenon from occurring. On a

larger scale, the mechanisms of this particular co-pathogen dynamic may further inform other co-infection scenarios in humans or other animals. The experimental hypothesis and specific aims to address these openings in knowledge will be discussed in the following chapters of this dissertation.

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Section 4 – Hypothesis and Specific Aims

Hypothesis

The synergistic pathogenesis of *Mycoplasma gallisepticum* and low-pathogenic avian influenza virus (LPAIV) is contingent upon the shared bacterial sialidase and viral neuraminidase activity. Co-infection of chickens with virulent or attenuated *M. gallisepticum* and LPAIV will induce differential pathological and transcriptional responses in the host at early time points compared to mono-infection. This co-infection will allow for the chronic persistence of attenuated *M. gallisepticum* mutants in vivo.

Specific Aims

Aim 1: Characterize the significance of sialic acids in *M. gallisepticum* adherence to host cells and effect of the viral neuraminidase inhibitor oseltamivir on *M. gallisepticum* neuraminidase activity.

Sub-aim 1A: Determine the role of sialic acid moieties in the cytadherance of *M. gallisepticum* to host cells.

Sub-aim 1B: Investigate the efficacy of oseltamivir on *M. gallisepticum* growth and neuraminidase activity and LPAIV H3N8 neuraminidase activity.

Aim 2: Evaluate the co-pathogenesis of *M. gallisepticum* and LPAIV *in vivo* within the natural chicken host.

Sub-aim 2A: Observe potential gross and histopathological differences in co-pathogenesis determined by the order of infection with each co-pathogen as well as a neuraminidase-negative *M. gallisepticum* mutant.

Sub-aim 2B: Utilize RNA-sequencing to characterize the chicken transcriptomic response to mono- and co-infection with *M. gallisepticum* and LPAIV.

Sub-aim 2C: Observe differences in the chronic persistence *in vivo* of attenuated *M. gallisepticum* mutants in a co-infection with LPAIV.

Aim 3: Evaluate the potential compensatory relationship between *M. gallisepticum* neuraminidase and LPAIV neuraminidase *in* vivo in the presence of the viral neuraminidase inhibitor oseltamivir.

Chapter 2 - The significance of sialic acids in *Mycoplasma gallisepticum* adherence to host cells and effect of the viral neuraminidase inhibitor oseltamivir on *M. gallisepticum* enzymatic activity.

Methods

-^{3}H-Labeled Mycoplasma Binding Assay: To investigate the importance of sialic acid moieties on *M. gallisepticum* binding to eukaryotic cells, *M. gallisepticum* R_{low} were grown in complete Hayflick's medium with 10 µCi/mL of ^{3}H-methyl thymidine (Perkin Elmer) at 37°C to mid-log phase. MRC-5 human lung fibroblasts and Madin-Darby Canine Kidney (MDCK) cell monolayers were cultivated in complete Eagle's Minimal Essential Medium (MEM) and seeded in 24-well tissue culture plates at a density of 1 x 10^{5} cells per well in triplicate. These cell lines are appropriate for use because MRC-5 cells effectively bind *M. gallisepticum in vitro* and MDCK cells are the conventional host cell for influenza A virus growth [1,2,3].

Wells of each cell type were pre-treated with Type VI neuraminidase (Sigma-Aldrich) for 30 minutes at 37°C in 5% CO_2 and washed. ^{3}H-labeled *M. gallisepticum* R_{low} cells was then washed and used to inoculate MRC-5 and MDCK monolayers and incubated for 1 hour at 37°C in 5% CO_2 and washed again at the end of the incubation. Bound *M. gallisepticum* and eukaryotic cells were then be lysed with 0.05 M NaOH and collected for reading in duplicate for counts per minute on the Beckman LS 3801 scintillation counter. A Student's T-Test was used to determine statistically significant

differences between neuraminidase treated and untreated control eukaryotic cell monolayers in their ability to bind *M. gallisepticum* at a significant level of $p < 0.05$.

-Mycoplasma Growth Inhibition Assay: To evaluate any effect of oseltamivir on the growth of *Mycoplasma gallisepticum in vitro*, cultures of *M. gallisepticum* R_{low} were grown to mid-log phase in Hayflick's medium and resuspended to a concentration of 1 x 10^8 CFU/mL in fresh Hayflick's medium. Oseltamivir carboxylate (Cayman Chemical) was dissolved in PBS, sterile-filtered and added to *M. gallisepticum* R_{low} at concentrations of 0.1 µM, 1 µM, 10 µM, 100 µM and 1 mM. Tetracycline was then added to *M. gallisepticum* R_{low} at concentrations of 5 mg/L and 10 mg/L in Hayflick's medium as a control for antibiotic growth inhibition, as well as untreated *M. gallisepticum* R_{low} included as a positive growth control. All cultures were incubated overnight at 37°C and growth was observed by color change in the medium compared to un-inoculated control medium.

-Mycoplasma Neuraminidase Inhibition Assay: In order to determine the potential effect of oseltamivir on *M. gallisepticum* neuraminidase activity *in vitro*, cultures of *Mycoplasma gallisepticum* R_{low} and Tn4001mod insertional mutant P1C5 were grown to mid-log phase in Hayflick's complete medium [4]. This mutant has been documented to be negative for neuraminidase activity and is attenuated *in vivo* [4]. The mutant P1C5 was grown in this medium supplemented with 200µg/mL of gentamicin for Tn4001mod transposon insertion maintenance. In addition, *Mycoplasma sturnii* UCMF was cultivated in Hayflick's complete medium for use as a neuraminidase negative

Mycoplasma in these assays [5]. Bacterial cells were then pelleted, washed, and resuspended in 1x PBS at 1 x 10^8 CFU/mL with concentrations of oseltamivir carboxylate (Cayman Chemical) at 0.1 μM, 10 μM, 100 μM and 1 mM with untreated suspensions of each culture included as a negative control and incubated for 30 minutes at 37°C. Each of these treatments were done in triplicate and seeded in a black-walled 96-well plate.

After incubation, Mycoplasma cells were then incubated with a buffer of sodium acetate and the fluorogenic substrate 20-(4-methy-lumbelliferyl)-a-D-N-acetylneuraminic acid (MUAN) (Sigma-Aldrich) and read on a fluorescent microplate reader at 360 nm excitation and 460 nm emission [6]. Fluorescence values were normalized by protein content and background fluorescence seen in neuraminidase-negative Mycoplasma *M. sturnii* UCMF. In order to determine statistically significant differences in neuraminidase activity among treatments, a one-way ANOVA with a Tukey's post-hoc test was utilized at a significance threshold of $p < 0.05$.

-Viral Neuraminidase Inhibition Assay: Analysis of the neuraminidase inhibitor, oseltamivir, and its ability to inhibit H3N8 (A/duck/Ukraine/1963) was performed *in vitro* with adaptations from the methods described by Eichelberger *et al*. [7]. The active metabolite form of oseltamivir, oseltamivir carboxylate (Cayman Chemical) was incubated on washed MDCK cells mono-layers seeded in a 96-well plate at concentrations ranging from 0 to 1000 nanomoles. These cell monolayers were then infected with H3N8 (A/duck/Ukraine/1963) at an MOI of 0.075 and 0.02 and incubated overnight at 37°C and 5% CO_2. After incubation, 25 μl of the supernatant was collected

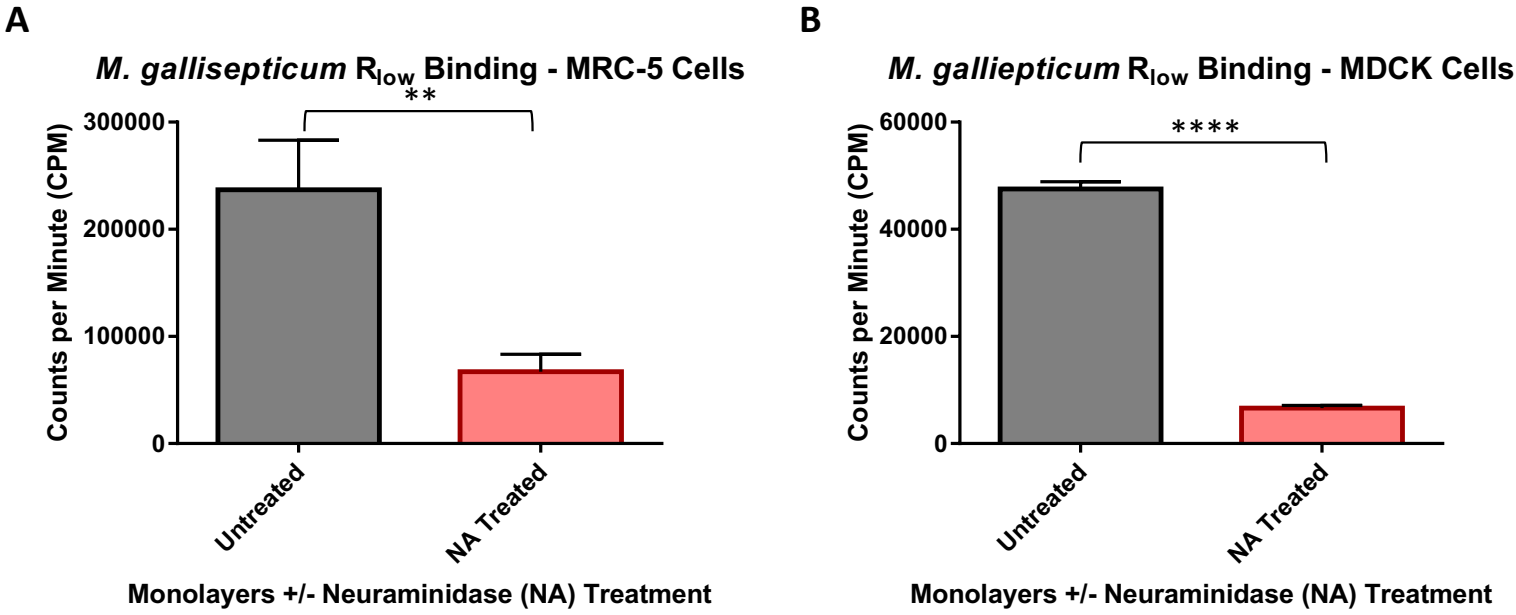
from each well and incubated with the fluorogenic substrate MUAN (Sigma-Aldrich) in a black-walled 96-well plate for 1 hour at 37°C. After the addition of a stop solution of 0.1 M glycine and 25% ethanol, the plate was read at 355 nm excitation and 460 nm emission on a fluorescent microplate reader. Statistically significant differences between neuraminidase activity levels were calculated using a mixed effects one-way ANOVA with Tukey's post-hoc test and a significance threshold of $p < 0.05$.

Results

In the case of both MRC-5 and MDCK cell monolayers, type VI neuraminidase pre-treatment significantly decreased ^{3}H-labeled *M. gallisepticum* R_{low} binding ($p < 0.01$ and $p < 0.0001$ respectively) (Figures 1A and 1B). This particular type VI neuraminidase is derived from *C. perfringens* and preferentially cleaves α-2,3 linked sialic acids over α-2,6 linked sialic acids (Sigma Aldrich). It can be surmised that these α-2,3 linked sialic acid moieties on the eukaryotic host cells are important in *M. gallisepticum* R_{low} cytadherance. These α-2,3 linked sialic acids are the primary binding target for avian influenza A viruses, which has great relevance in the context of an avian influenza virus and *M. gallisepticum* co-infection scenario in an avian system [8].

Figure 1 – ^{3}H-labeled *M. gallisepticum* binding to MRC-5 (Figure 1A) and MDCK (Figure 1B) monolayers with and without neuraminidase (NA) treatment quantified by counts per minute (CPM). Error bars represent SEM. Significant differences calculated

using an Unpaired T-test. Statistical significance markers ** indicate $p < 0.01$ and **** indicate $p < 0.0001$.

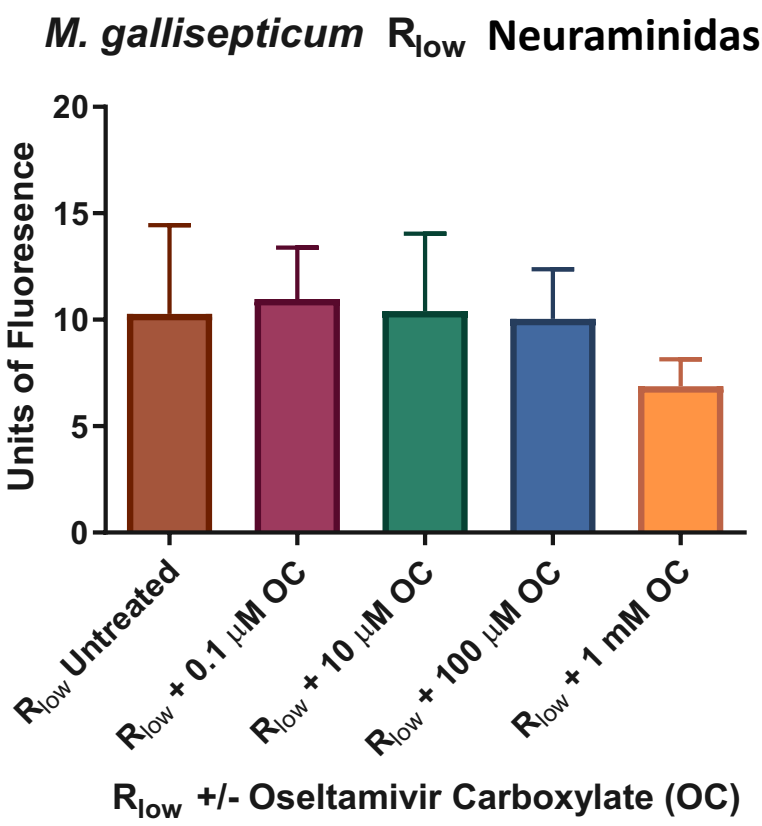


At all concentrations of oseltamivir carboxylate tested (0.1 µM, 1 µM, 10 µM, 100 µM and 1 mM), there was no inhibition of *M. gallisepticum* R_{low} growth as indicated by Hayflick's medium color change when compared to *M. gallisepticum* R_{low} grown in the absence of any antibiotic treatment (data not shown). This suggests that any effects on *M. gallisepticum* R_{low} neuraminidase activity in subsequent experiments using oseltamivir can be attributed to effects on the neuraminidase enzyme and not the growth kinetics of *M. gallisepticum*.

When incubated with oseltamivir carboxylate, there were no significant differences between *M. gallisepticum* R_{low} neuraminidase activity among un-treated control R_{low} and R_{low} treated with 0.1 µM, 10 µM, 100 µM and 1 mM oseltamivir carboxylate (Figure 2). These concentrations range above and below the concentration

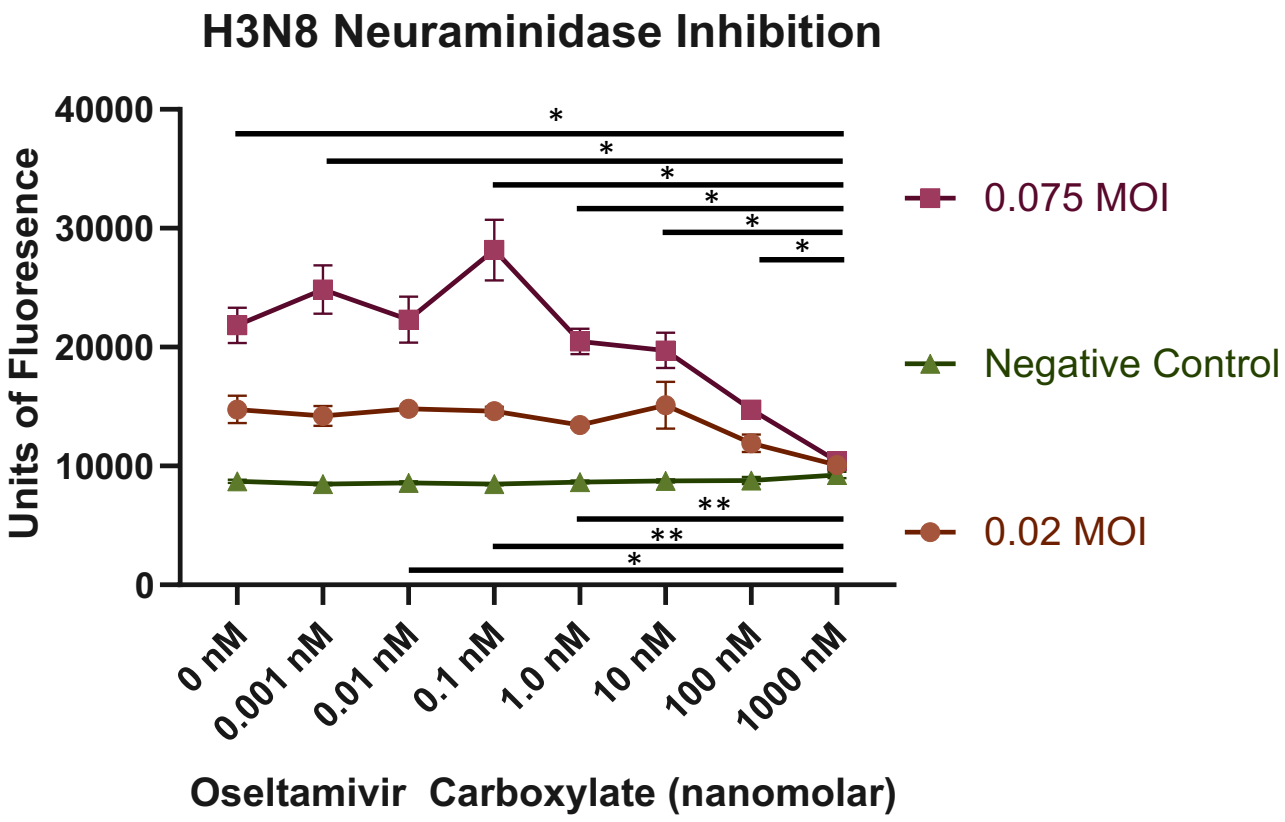
of oseltamivir used commonly to inhibit neuraminidase activity of other bacterial organisms, such as *Streptococcus pneumoniae* [9]. This indicates that oseltamivir carboxylate does not have an inhibitory effect on the neuraminidase activity of *M. gallisepticum* R_{low}. In a co-infection environment with influenza A virus, oseltamivir inhibition would likely act solely on the influenza virions and open the possibility for *M. gallisepticum* neuraminidase activity to compensate or augment the neuraminidase activity of influenza. Such a mechanism could contribute to the exacerbated pathogenesis seen in models of *M. gallisepticum* and LPAIV co-infection.

Figure 2 – *M. gallisepticum* neuraminidase activity with and without treatment with oseltamivir carboxylate (OC). No significant differences found using One-Way ANOVA and Tukey's Post-Hoc test.



In contrast to the data in Figure 2 pertaining to *M. gallisepticum* neuraminidase activity, oseltamivir carboxylate does effectively inhibit H3N8 neuraminidase activity (Figure 3). At an MOI of 0.02, H3N8 neuraminidase activity was significantly different between the highest dose, 1000 nM, and 0.01 nM, 0.1 nM, and 1.0 nM oseltamivir carboxylate treatment (Figure 3). This effect was even more pronounced at an MOI of 0.075, in that H3N8 neuraminidase activity was significantly different between the highest dose of 1000 nM and 0 nM, 0.001 nM, 0.1 nM, 1.0 nM, 10 nM, and 100 nM (Figure 3). These concentrations represent a spectrum of relevant neuraminidase inhibitor concentrations used in neuraminidase inhibition *in vitro* assays [7].

Figure 3 - LPAIV H3N8 neuraminidase activity when incubated with oseltamivir carboxylate (0 to 1000 nanomolar). MDCK cells were infected with H3N8 at two MOI (0.075 and 0.02), as well as MDCK monolayers infected with no virus as a negative control. Significant differences in neuraminidase activity calculated using mixed-effects one-way ANOVA and Tukey's post-hoc test. Statistically significant differences are notated as * = $p < 0.05$ and ** = $p < 0.005$. The statistical markers on the upper-most portion of the figure pertain to cells infected at an MOI of 0.075, and the markers on the bottom portion of the figure relate to cells infected at an MOI of 0.02.



Discussion

The data presented above elude to possible factors contributing to the co-pathogenesis of LPAIV and *M. gallisepticum* in terms of host cell tropism and possible compensatory neuraminidase activity. The significant disruption in cytadherance to host cells by *M. gallisepticum* in the absence of α-2,3 linked sialic acids is a novel finding. In the context of a co-infection with LPAIV, this result holds even more significance in that LPAIVs preferentially bind host tissues at α-2,3 linked sialic acid moieties common in the avian respiratory and gastrointestinal tract [10].

A common host cell ligand could indicate potential competition between *M. gallisepticum* and LPAIV for proximity to the host cell. However, the data from *in vitro* and *in vivo* studies examining these co-pathogens indicates that there is more benefit to replication and persistence when these two pathogens are present over a hampering of one's survival in favor of the other [11,12,13].

The shared importance of α-2,3 linked sialic acids in these co-pathogens interaction with the host more likely indicates that these two pathogens may bind in close proximity to one another on host tissues. This proximity facilitates an array of potential mechanisms of mutual benefit during co-pathogenesis. These mechanisms could include host metabolites, the availability of host cell receptors, or the shared activity of enzymes such as the viral and bacterial neuraminidases.

As seen in other viral and bacterial co-pathogen relationships, neuraminidase activity can be pivotal in the mutualistic dynamic during the disease process. For instance, *Streptococcus pneumoniae* has potent neuraminidase activity that can rescue

influenza viral replication in the presence of viral neuraminidase inhibitors [14]. The ability of *M. gallisepticum* and LPAIV to attach to host cells in potentially close-quarters would allow for this same dynamic of neuraminidase activity compensation during infection.

This hypothesis garners further merit in that *M. gallisepticum* R_{low} is not susceptible to oseltamivir carboxylate neuraminidase inhibition, whereas LPAIV H3N8 neuraminidase is susceptible to this inhibitor at a range of concentrations. In a case of this co-infection in an avian population, neuraminidase inhibitor treatment could be rendered useless if *M. gallisepticum* neuraminidase can effectively compensate for LPAIV and further the progression of disease.

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Chapter 3 – Transcriptional and pathological host responses to co-infection with virulent or attenuated *Mycoplasma gallisepticum* and low pathogenic avian influenza A virus in chickens.

Ammended Version Submitted to Infection and Immunity for Publication (IAI00607-19)

Authors

J.A. Canter [a], E.R. Tulman [a], J. Beaudet [a,b], D. Lee [a], M. May [c], S.M. Szczepanek [a], and S.J. Geary [a]*

Department of Pathobiology and Veterinary Sciences and the Center of Excellence for Vaccine Research, University of Connecticut, Storrs, Connecticut, USA [a]; Department of Allied Health Sciences, University of Connecticut, Storrs, Connecticut, USA [b]; Department of Biomedical Sciences, University of New England College of Medicine, Biddeford, Maine, USA [c].

*Corresponding author

Abstract

The avian pathogen *Mycoplasma gallisepticum*, the etiologic agent of chronic respiratory disease in chickens, exhibits enhanced pathogenesis in the presence of a co-pathogen such as low-pathogenic avian influenza virus (LPAIV). To further investigate the intricacies of this co-pathogenesis, chickens were mono- or co-infected with either virulent *M. gallisepticum* strain R_{low}, attenuated *M. gallisepticum* mutant

P1C5, or LPAIV H3N8 (A/duck/Ukraine/1963) and assessed for tracheal histopathology, pathogen load, and transcriptomic host response to infection using RNA-sequencing. Chickens co-infected with *M. gallisepticum* R_{low} followed by H3N8 exhibited significantly more severe tracheal lesions and mucosal thickening in response to infection than chickens infected with H3N8 alone. Viral load was also significantly increased in this group over chickens who were infected first with H3N8 and subsequently with *M. gallisepticum* R_{low}. The attenuated *M. gallisepticum* mutant P1C5, previously shown to be cleared 14 days post-infection, was able to persist 6 days post-infection in the presence and absence of co-infection with H3N8. The transcriptional response to mono- and co-infection with *M. gallisepticum* and LPAIV highlighted the involvement of differential expression of genes such as TLR4, TLR15, TLR21, IL-1β, IRF4, MMP1, and MMP9. Pathway and gene ontology analysis of these differentially expressed genes suggests that co-infection with virulent *M. gallisepticum* and LPAIV induces a downregulation of ciliary activity *in vivo* and alters the multiple immune-related signaling cascades. These data aid in the understanding of the relationship between *M. gallisepticum* and LPAIV during co-pathogenesis in the natural host and may contribute to the further understanding of co-pathogen infections of humans and other animals.

Introduction

The bacterial pathogen *Mycoplasma gallisepticum* causes significant avian diseases such as sinusitis in turkeys, conjunctivitis in passerines, and chronic respiratory disease in chickens [1,2]. Pathogenesis of *M. gallisepticum* in chickens

involves severe inflammation in components of the respiratory system, as well as deleterious effects on egg production in these birds, making *M. gallisepticum* a pathogen of great relevance and concern to the agricultural industry [1]. Infection of chickens with virulent *M. gallisepticum* R_{low} has been shown to induce a dysregulated immune response in the chicken host with differential gene expression in the trachea peaking 3 days post-infection over the first 7 days [3]. Toll-like receptor (TLR) responses, most notably TLR4 and TLR15, were significantly upregulated in the trachea during infection, as were IL-1β, MMP7, and an array of inflammatory cytokines and chemokines [3]. An additional report demonstrated that the lipoproteins of *M. gallisepticum* stimulate a TLR2-mediated response in primary chicken tracheal epithelial cells [4].

Another avian pathogen of great importance is avian influenza A virus. Avian influenza viruses occur in two main categories; highly-pathogenic avian influenza viruses (HPAIV) and low-pathogenic avian influenza viruses (LPAIV) [5]. HPAIV causes severe disease in a multitude of avian species with an extremely high mortality rate [6]. In contrast, LPAIV causes little-to-no disease in birds and, without rigorous surveillance, could go unnoticed in wild or domestic populations of birds [6].

Co-infections consisting of *M. gallisepticum* and LPAIV in chickens exacerbates disease compared to either pathogen in isolation]. Chickens challenged with both *M. gallisepticum* strain 1226 and LPAIV H3N8 (A/mallard/Hungary/19616/07) showed significantly more severe clinical signs and increased recovery of *M. gallisepticum* [7]. Co-infection with these two pathogens also yields significantly more severe clinical scores of gross and histopathologic lesions of infected chickens [8]. Ex vivo

experiments with *M. gallisepticum* strain S6 and H9N2 (A/chicken/Saudi Arabia/CP7/1998) in an avian tracheal organ culture system showed significant differences in replication of *M. gallisepticum* and H9N2 between mono- and co-infected tracheas [9]. Ciliostasis was also observed in these co-infected tracheal cultures at a significantly higher rate and severity over mono-infected tracheas as well [9].

A possible mechanism underlying this co-pathogenesis phenomenon is the common functionality of the glycosidic enzymes neuraminidase of influenza A virus, and sialidase of *M. gallisepticum*. Influenza A virus utilizes neuraminidase to cleave *N*-acetylneuraminic acids, or sialic acids, on host cells to release progeny virions during infection. In *M. gallisepticum*, sialidase performs this same enzymatic reaction and transposon insertional disruption of this gene in virulent *M. gallisepticum* R_{low} results in attenuation of the pathogen *in vivo* [10,11]. The true role of sialidase in the life cycle and pathogenesis of *M. gallisepticum* is still yet to be elucidated. For the remainder of this manuscript, both influenza A virus neuraminidase and Mycoplasma sialidase will be referred to as neuraminidases for the sake of continuity.

Instances of influenza A virus co-infection with bacterial pathogens in humans, such as *Streptococcus pneumoniae*, yield evidence that the neuraminidases of influenza virus and *S. pneumoniae* work in concert during infection to enhance disease [12,13]. In the presence of a viral neuraminidase inhibitor zanamivir, *S. pneumoniae* neuraminidase can compensate for the hampered neuraminidase activity of influenza virus to enhance viral replication and further co-pathogenesis in mice [14].

In the case of *M. gallisepticum* and LPAIV co-infection, there is strong evidence that the presence of both pathogens enhances various markers of disease over

infection with either pathogen alone. However, the host-pathogen dynamics involved in this co-pathogenesis relationship remain unexplored. It is our hypothesis that *M. gallisepticum* neuraminidase works to support LPAIV neuraminidase during co-infection. We also hypothesize that co-infection of chickens with *Mycoplasma gallisepticum* and LPAIV H3N8 results in more severe disease than mono-infection, and that this difference in pathogenesis will yield changes in pathologic lesions, pathogen load, and transcriptional changes in the host. To examine this, we utilized an *in vivo* infection model in chickens, a natural host of both *M. gallisepticum* and LPAIV. In addition, we hypothesize that co-infection with LPAIV H3N8 would cultivate an environment to sustain the chronic persistence of an attenuated *M. gallisepticum* mutant *in vivo*.

The co-pathogenesis of *M. gallisepticum* and LPAIV is of great relevance to the agricultural industry for the prevention and treatment of viral and bacterial infections in avian flocks [11]. Either pathogen alone may go un-detected in a population of birds for a sufficient period of time to set the stage for severe losses due to later infection with the other pathogen. The dynamics involved in this system of co-infection may also prove beneficial in understanding the factors contributing to instances of viral and bacterial co-pathogenesis in other animals and humans.

Methods

-Animals: For each study, four-week old female specific pathogen-free (SPF) White Leghorn chickens (SPAFAS, North Franklin, CT, USA) were procured and separated randomly into experimental groups for placement in HEPA-filtered housing isolators. These chickens then acclimated in this habitat for a period of one week before the start

of the experiment. For this acclimation period, as well as the remainder of the experiment, the chickens were maintained on non-medicated feed and water ad libitum. These animal experiments were performed with approval from the University of Connecticut Institutional Animal Care and Use Committee under protocol #A18-057.

-***Mycoplasma gallisepticum*** **and LPAIV Preparation**: Influenza A virus isolate H3N8 (A/duck/Ukraine/1963) (BEI Resources) was used to inoculate 10-day old SPF embryonated chicken eggs (SPAFAS, North Franklin, CT, USA) at 10-fold dilutions of viral stock [15,16]. After 48 hours, allantoic fluid was collected from these eggs and viral titer was determined by $TCID_{50}$ on Madin Darby Canine Kidney (MDCK) cells using the Reed-Meunch method [15,16]. Viral stocks were frozen at -80° C and diluted in complete Hayflick's medium to a concentration of 5 x 10^6 $TCID_{50}$/200 µL at the time of use as an experimental inoculum.

Mycoplasma gallisepticum strain R_{low} passage 17 was propagated from stock cultures frozen at -80°C in complete Hayflick's medium overnight at 37°C until mid-log phase. The attenuated *M. gallisepticum* tn4001 mutants P1C5 and P1H9 were also grown from frozen stock cultures in complete Hayflick's medium with the addition of 150 µg/mL for the maintain the position of tn4001 transposon insertion [11,17]. Mycoplasma cultures were quantified by optical density at 620 nm and then centrifuged for 10 minutes at 10,000 x g to pellet the cultures. Mycoplasma pellets were then resuspended in fresh complete Hayflick's medium to a density of 1 x 10^8 CFU/200 µL to create the experimental inoculum for each of the chicken infection experiments.

-Chicken Infection Experiments: 5 week old chickens were challenged intratracheally by pipetting 200 µL of either 1 x 10^8 CFU *M. gallisepticum* R_{low}, 1 x 10^8 CFU *M. gallisepticum* mutant P1C5, 5 x 10^6 $TCID_{50}$ LPAIV H3N8, or mock-challenged with fresh complete Hayflick's medium according to the following schedule (n = 5 per group):

Experiment #1		
Primary Infection (Day 0)	**Secondary Infection (Day 3)**	**Sacrifice (Day 6)**
Mock (Hayflick's medium)	*M. gallisepticum* R_{low}	
H3N8	*M. gallisepticum* R_{low}	
M. gallisepticum R_{low}	H3N8	
M. gallisepticum P1C5	H3N8	
Mock (Hayflick's medium)	H3N8	

In two companion challenge studies, additional set of chickens (n = 5 per group in experiment #2, n = 10 in experiment #3) were challenged as described above in the following groups:

Experiment #2	
Primary Infection (Day 0)	**Sacrifice (Day 6)**
M. gallisepticum R_{low}	
M. gallisepticum P1C5	
Mock (Hayflick's medium)	

Experiment #3			
Primary Infection (Day 0)	**Secondary Infection (Day 3)**		**Sacrifice (Day 14)**
M. gallisepticum P1C5	Mock (Hayflick's medium)		
M. gallisepticum P1C5	H3N8		
M. gallisepticum P1H9	Mock (Hayflick's medium)	**Sacrifice (Day 7)**	
M. gallisepticum P1H9	H3N8		

Another attenuated mutant, *mslA* mutant P1H9, was incorporated into this model of co-infection as a means to compare changes in *M. gallisepticum* mutant co-infection over the course of disease and be able to attribute the possible changes in

pathogenesis to the specific relationship between P1C5 and LPAIV neuraminidase activity [17]. The mutant P1C5 persists in the trachea of chickens 6 days post-infection, however the previous studies utilizing the mutant P1H9 only examined Mycoplasma recovery 14 days post-infection [17].

- **Sample Collection, Mycoplasma Recovery and RNA Extraction**: In each of the above experiments, all chickens from each of the groups were humanely sacrificed by cervical dislocation and the tracheas were excised. Three tracheal rings from each chicken were taken from the proximal, middle, and distal portion of each trachea and fixed in neutral buffered formalin for pathological assessments. An additional ring was taken from the distal end of the trachea of each bird and placed into complete Hayflick's medium for mycoplasma recovery and incubated for 3 hours at 37°C. After incubation, these cultures were filtered through 0.45 µm filter and diluted 10-fold for color changing unit (CCU) quantification, as done previously [18]. Statistically significant differences among groups for *M. gallisepticum* CCU recovery were calculated using using a Kruskal-Wallis ANOVA On Ranks with Dunn's Post-Hoc test (GraphPad Prism 8.0).

The remaining sections of excised tracheas from each bird were washed with 1 mL of TriZol (Zymo Research, Carlsbad, CA, USA) pipetted 4 times through the lumen for RNA collection as described previously [18]. RNA was extracted from these TriZol washes using the Zymo DirectZol RNA MiniPrep Kit (Zymo Research, Carlsbad, CA, USA) and quantified using a Qubit 2.0 Fluorometer (ThermoFisher Scientific).

-Tracheal Thickness and Lesion Scoring: Tracheal rings were embedded and sectioned for hematoxylin and eosin (H&E) staining as described previously [19]. These rings were measured for tracheal thickness and lesions were scored by an AAVP board-certified veterinary pathologist. These lesions were scored on a scale of 0 to 4, denoting no inflammation, mild inflammation, moderate inflammation, marked inflammation, and severe inflammation, respectively, as described previously [19]. Significant differences among challenge groups for tracheal thickness measurements were calculated using a one-way analysis of variance (ANOVA) and Tukey's Post-Hoc test and differences in tracheal lesion scores were calculated using a Kruskal-Wallis ANOVA On Ranks with Dunn's Post-Hoc test (GraphPad Prism 8.0).

-**Viral Load Quantification:** RNA collected from the tracheas of infected chickens in experiment #1 was assayed using qRT-PCR of the influenza matrix gene in a 25 µL reaction volume (Applied Biosystems 75000 Fast Real-Time PCR System) by the Connecticut Veterinary Medical Diagnostic Laboratory. Cycling conditions for this reaction were 45.0°C for 10 minutes, 95.0°C for 10 minutes, 94.0°C for 1 second, cycled at this step 45 times before a final step at 60.0°C for 30 seconds. A standard curve of H3N8 (A/duck/Ukraine/1963) RNA was used in 10-fold dilutions for calculation of viral genomes per microliter.

-Illumina Sequencing: RNA extracted from tracheal washes of each bird from experiment #1 were used as templates for cDNA library synthesis using the Illumina TruSeq Stranded mRNA Library Preparation Kit (Illumina, San Diego, CA). Each library

was quantified and assessed for quality using the Agilent TapeStation 2200 (Agilent Technologies). These libraries were then normalized and pooled for sequencing on the Illumina NextSeq 500 (Illumina, San Diego, CA) with paired-end, 75 base pair reads with 5 to 10 million reads per sample [3].

-RNA-seq Analysis: Read data from each bird sample in the Fastq format were mapped to the *Gallus gallus* reference genome using the TopHat package and Bowtie2 engine to produce sequence alignments [20]. Alignment files were pooled by experimental infection groups using Cufflinks to map to reference transcript files. Cuffdiff was utilized to determine expression values and statistically significant differences of these values between experimental infection groups. These expression values per transcript are normalized within group via calculation of fragments per kilobase per exon per million fragments mapped (FPKM). Benjamini-Hochberg analysis then generates a p-value for statistical significance also accounting for a false discovery rate of <5% to yield a q-value of significance. Fold change differences between experimental infection groups were calculated by a $\log_2$ calculation of FPKM values from each group [20]. Significantly differentially expressed genes with greater than, or equal to, a positive or negative 2 $\log_2$ fold change will be displayed for between-group comparisons.

-Pathway and Functional Gene Ontology Analysis: Significantly differentially expressed genes (DEGs) were used as an input for analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 to assemble

these DEGs into biological pathways defined by the Kyoto Encyclopedia of Genes and Genomes (KEGG) and functional gene ontologies using GO Term BP Direct [21].

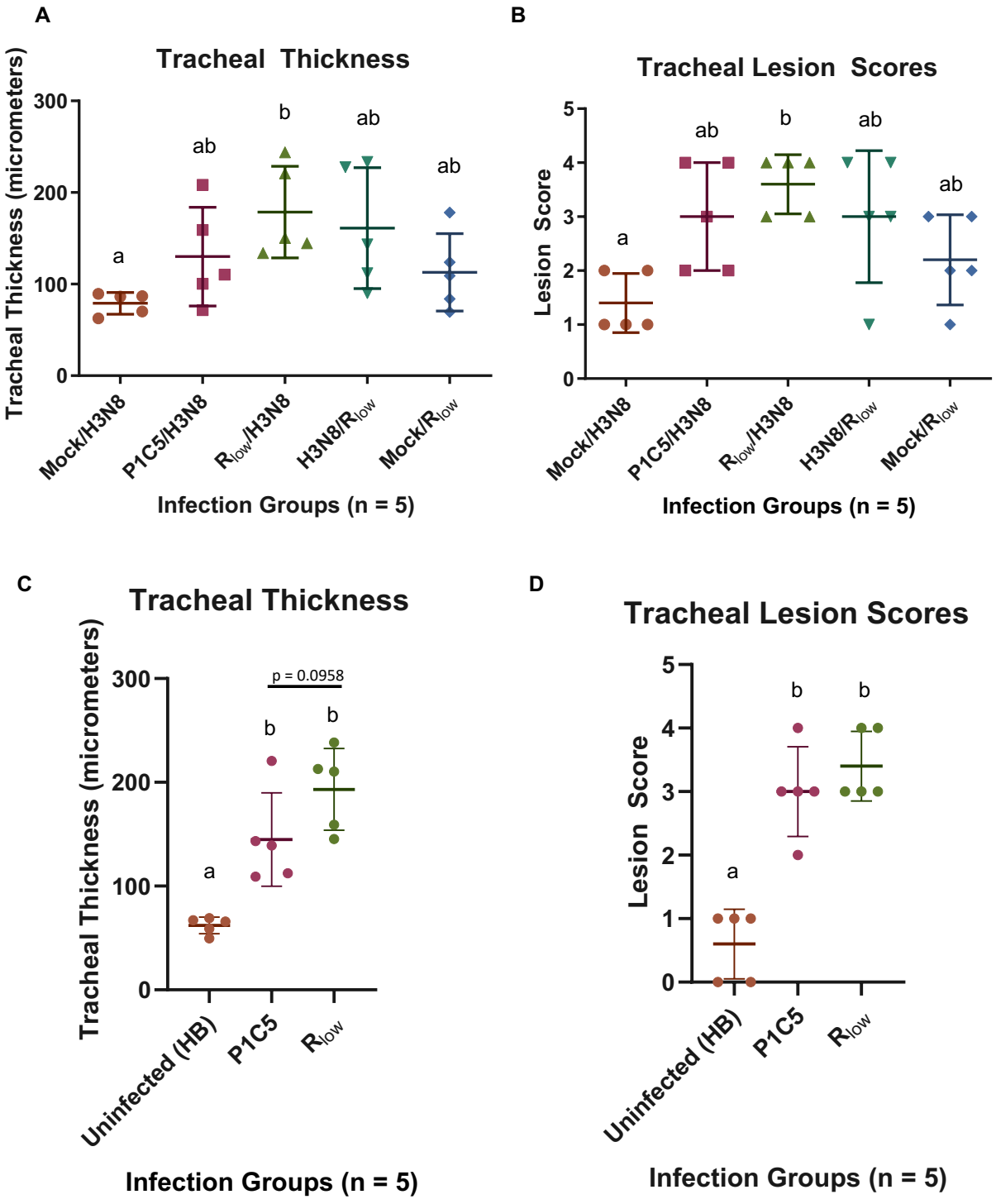
Results

-Tracheal Thickness and Lesion Scores: Significant differences were found in the tracheal mucosal thickness ($p < 0.05$) between Mock/H3N8 and *M. gallisepticu*m R_{low}/H3N8 infected chickens in experiment #1 (One-Way ANOVA with Tukey's Post-Hoc test). Significant differences were observed in histopathologic tracheal lesion scores ($p < 0.05$) between Mock/H3N8 and *M. gallisepticu*m R_{low}/H3N8 infected chickens of experiment #1 (Kruskal-Wallis ANOVA On Ranks with Dunn's Post-Hoc test) (Figures 1A and 1B). Although not statistically significant at this timepoint, tracheal thickness ($p = 0.3901$) exhibits a trend of increased severity in the H3N8/*M. gallisepticum* R_{low} group compared to the Mock/*M. gallisepticum* R_{low} group (Figures 1A and 1B).

In experiment #2, significant differences exist between mock infected chickens and R_{low} infected chickens as well as mock infected and P1C5 infected chickens for both tracheal thickness (Figure 1C) and tracheal lesion scores (Figure 1D). These data indicate that although no lesions or live *M. gallisepticum* P1C5 were observed 14 days post-infection in previous studies from our laboratory, this mutant appears to persist in the chicken host 6 days post-infection and induces a moderate pathologic response in the lumen of the trachea [11].

Figure 1 – Tracheal lesion scores (A) and thickness measurements (B) from *M. gallisepticum* and H3N8 mono- and co-infected chickens. Statistically significant

differences among group tracheal lesion scores were calculated using a Kruskal-Wallis ANOVA On Ranks with Dunn's Post-Hoc test. Statistically significant differences among group tracheal thickness measurements were calculated using a One-Way ANOVA with Tukey's Post-Hoc test. Significant differences are denoted using "a" and "b" indicating a significant difference of $p < 0.05$. Tracheal histopathology (C and D) from experiment #2. Statistically significant differences among tracheal thickness for experimental groups were calculated using a One-Way ANOVA with Tukey's Post-Hoc Test and are indicated by ** = $p < 0.05$, *** = $p < 0.0005$. For tracheal lesion scores in experiment #2, significant differences were calculated using a Kruskal-Wallis ANOVA On Ranks with Dunn's Post-Hoc test and using "a" and "b" indicating a significant difference of $p < 0.01$ and $p < 0.05$.



-Mycoplasma and Viral Load *M. gallisepticum* was recovered from birds in all groups that received an *M. gallisepticum* inoculation in experiment #1 (Figure 2A). The

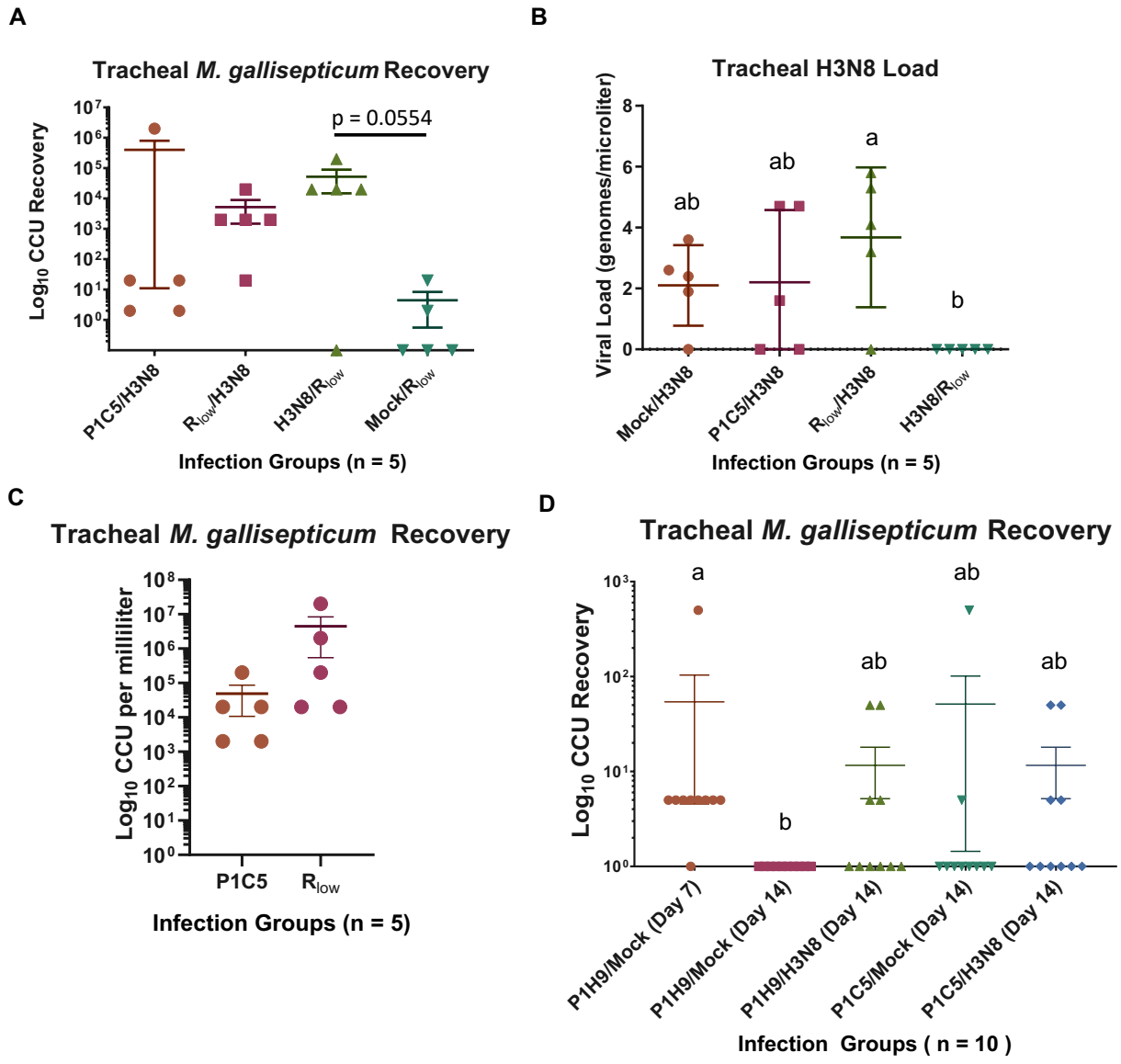
bacterial loads recovered from H3N8/*M. gallisepticum* R_{low} infected chickens were significantly higher than the Mock/*M. gallisepticum* R_{low} group ($p < 0.05$) (Kruskal-Wallis ANOVA On Ranks with Dunn's Post-Hoc test) (Figure 2A). Although tracheal pathology did not differ significantly between the H3N8/*M. gallisepticum* R_{low} group and Mock/*M. gallisepticum* R_{low} group (Figure 1A and 1B), *M. gallisepticum* was recovered from 80% of chickens within the H3N8/*M. gallisepticum* R_{low} group (Figure 2A), 20% of chickens in the Mock/*M. gallisepticum* R_{low} group and , 100% from chickens in the *M. gallisepticum* R_{low}/H3N8 group (Figure 2A).

In a similar vein, H3N8 was detected in chickens within all groups receiving an inoculation with H3N8 in experiment #1, with the exception of chickens in the H3N8/*M. gallisepticum* R_{low} infection group (Figure 2B). H3N8 was detected in 80% of chickens in the Mock/H3N8 group and the *M. gallisepticum* R_{low}/H3N8 group, both having been challenged with H3N8 for the final3 days of the experiment. H3N8 was not detected in the H3N8/*M. gallisepticum* R_{low} group after 6 days of H3N8 infection. H3N8 load was significantly higher in the *M. gallisepticum* R_{low}/H3N8 group over the H3N8/*M. gallisepticum* R_{low} group of experiment #1 ($p < 0.01$, One-Way ANOVA with Tukey's Post-Hoc test) (Figure 2B).

In experiment #2, *M. gallisepticum* was recovered from the tracheae of all *M. gallisepticum* infected birds for both *M. gallisepticum* R_{low} and P1C5 (Figure 2C). This bacterial recovery follows a similar pattern to the tracheal histopathology displayed in Figure 1, in that *M. gallisepticum* R_{low} was the most abundant in the trachea at 6 days post-infection and induced more severe tracheal lesions and thickening than the mutant P1C5, which was recovered at a lesser abundance at that time (Figure 2C).

Experiment #3 demonstrated that *M. gallisepticum* was recovered from all *M. gallisepticum* P1H9 infected chickens, with the exception of P1H9/Mock infected birds sacrificed at 14 days post-infection (Figure 3). The difference in *M. gallisepticum* recovery between these two groups is statistically significant ($p < 0.001$) (Kruskal-Wallis ANOVA On Ranks with Dunn's Post-Hoc test). P1H9 was previously shown to fail to persist at 14 days post-infection [17], however these data show that this attenuated mutant does survive in the chicken trachea at 7 days post-infection in 90% of infected chickens. Persistence in the host trachea to 14 days post-infection was partially rescued only in the presence of a co-infection with H3N8 in 40% of infected chickens (Figure 2D).

Figure 2 – Recovery of *M. gallisepticum* (A) and H3N8 (B) from the tracheas of mono- and co-infected chickens of experiment #1. *M. gallisepticum* recovery (C) from the tracheas of infected chickens of experiment #2 and experiment #3 (D). Bacterial recovery was quantified using color changing unit (CCU) dilutions. Statistically significant differences in *M. gallisepticum* recovery were determined using a Kruskal-Wallis ANOVA On Ranks with Dunn's Post-Hoc test and denoted using "a" and "b" indicating a significant difference of $p < 0.05$ (C). Significany differences in H3N8 load were calculated using One-Way ANOVA with Tukey's Post-Hoc test and denoted using "a" and "b" indicating a significant difference of $p < 0.05$ (B).



-Significantly Differentially Expressed Gene (DEG) Analysis: CuffDiff differential expression analysis between challenge groups of experiment #1 determined that there are 260 significant DEGs increased in expression and 286 significant DEGs decreased in expression between *M. gallisepticum* R_{low}/H3N8 and Mock/H3N8 infected chickens. Lists of DEGs between experimental groups of experiment #1 with a greater than 2 log_2 fold change can be found in Supplemental Tables 10 through 14.

-Toll-Like Receptor (TLR) Genes

Table 1:

Significantly Differentially Expressed Toll-Like Receptors > 2 Fold Change (FC)				
Experimental Group Comparison	**Ensemble Gene ID**	**Gene Name**	**Log_2 FC**	**q Value**
P1C5/H3N8 vs. Rlow/H3N8	ENSGALG00000007001	TLR4	3.020	0.0044
P1C5/H3N8 vs. Rlow/H3N8	ENSGALG00000008166	TLR15	2.981	0.0044
Mock/H3N8 vs. Mock/Rlow	ENSGALG00000008166	TLR15	-2.028	0.0115
Mock/H3N8 vs. Rlow/H3N8	ENSGALG00000000774	TLR21	-2.100	0.0184
Mock/H3N8 vs. P1C5/H3N8	ENSGALG00000000774	TLR21	-2.346	0.0097

Various TLR genes are significantly differentially expressed between groups of infected chickens in experiment #1. TLR4, which recognizes bacterial lipopolysaccharide, is significantly upregulated between *M. gallisepticum* P1C5/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens (Table 1). This increased expression is unusual due to the absence of lipopolysaccharide in *Mycoplasmas*, however TLR4 has also been shown to be upregulated in response to *M. gallisepticum* R_{low} infection *in vivo* [3].

TLR15, an avian and reptile-specific TLR, is shown to be upregulated in response to yeast and bacterial pathogen-associated molecular patterns (PAMPS) [25]. TLR15 is increased in expression between *M. gallisepticum* P1C5/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens and decreased between Mock/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens (Table 1).

TLR21 is decreased in expression between Mock/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens as well as Mock/H3N8 and *M. gallisepticum* P1C5/H3N8 infected chickens (Table 1). TLR21 is an avian functional homolog of human TLR9 and

recognizes CpG oligodeoxynucleotides [24]. The suppression of these two TLR transcripts during mono- or co-infection with *M. gallisepticum* R_{low} compared to mono-infection with H3N8 suggests potential immune signaling dysregulation induced by *M. gallisepticum* in this model.

Table 2:

Significantly Differentially Expressed Genes > 2 Fold Change (FC)				
Experimental Group Comparison	**Ensemble Gene ID**	**Gene Name**	**Log2 FC**	**q Value**
P1C5/H3N8 vs. Rlow/H3N8	ENSGALG00000019061	MMP1	3.801	0.0044
Mock/H3N8 vs. P1C5/H3N8	ENSGALG00000019061	MMP1	-2.809	0.0097
Mock/H3N8 vs. Mock/Rlow	ENSGALG00000019061	MMP1	-2.054	0.0115
P1C5/H3N8 vs. Rlow/H3N8	ENSGALG00000006992	MMP9	3.704	0.0044
Mock/H3N8 vs. P1C5/H3N8	ENSGALG00000006992	MMP9	-2.224	0.0097
P1C5/H3N8 vs. Rlow/H3N8	ENSGALG00000012830	IRF-4	2.154	0.004
P1C5/H3N8 vs. Rlow/H3N8	ENSGALG00000000534	IL-1BETA	3.508	0.0044
Mock/H3N8 vs. P1C5/H3N8	ENSGALG00000000534	IL-1BETA	-2.262	0.0097

Matrix Metalloproteinase (MMP) Genes

The expression of MMP genes is differentially affected among comparisons of groups in experiment #1. MMP1 and MMP9 are both decreased in expression between Mock/H3N8 and *M. gallisepticum* P1C5/H3N8 infected chickens (Table 2). In addition, MMP1 is upregulated between *M. gallisepticum* P1C5/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected birds and downregulated between Mock/H3N8 and Mock/*M. gallisepticum* R_{low} infected birds (Table 2). MMP9 is upregulated between *M. gallisepticum* P1C5/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens (Table 2). Differences in the expression of these two MMP genes may indicate alterations in the rates of tissue damage and repair among experimental groups. Upregulation of these

MMPs in *M. gallisepticum* R_{low}/H3N8 infected chickens associates with the enhanced tracheal histopathology seen in Figure 1.

Interferon and Interleukin Genes

Interferon regulatory factor 4 (IRF4) is significantly upregulated between *M. gallisepticum* P1C5/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens (Table 2). This indicates an activation of the type I interferon response to influenza virus in chickens co-infected with virulent *M. gallisepticum* R_{low}.

Interleukin 1 beta (IL-1β) is significantly upregulated between *M. gallisepticum* P1C5/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens but downregulated between Mock/H3N8 and *M. gallisepticum* P1C5/H3N8 infected birds (Table 2). This differential regulation of IL-1β expression could indicate an enhanced activation of the NLRP3 inflammasome during co-infection with virulent *M. gallisepticum* R_{low} and overall increased inflammatory signaling unique to the presence of a pathogenic *M. gallisepticum*.

-Functional Gene Ontology:

Between Mock/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens these DEGs can be grouped into an array of functional pathways. Significant DEGs decreased in expression between these two challenge groups belong mostly to functional pathways involved in cilium organization and macromolecule metabolic processes (Supplemental Table 1). Significant DEGs increased in expression belong mainly to positive regulation of the immune system processes (Supplemental Table 2). Between *M. gallisepticum*

P1C5/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens, pathways increased in expression belong foremost to response to lipopolysaccharide and signal transduction (Supplemental Table 3), and pathways decreased in expression belong mainly to cellular component assembly involved in morphogenesis, microtubule-based movement and cilium organization (Supplemental Table 4).

-Pathway Analysis: KEGG pathway analysis of significant DEGs between experimental groups illuminated key pathways differentially regulated during mono- and co-infection. The toll-like receptor, phagosome, and cytokine-cytokine receptor pathways are significantly downregulated between Mock/H3N8 and Mock/*M. gallisepticum* R_{low} infected chickens as well as between Mock/H3N8 and *M. gallisepticum* P1C5/H3N8 infected chickens (Supplemental Tables 6 and 7). Metabolic pathways are significantly downregulated between the groups stated above as well as Mock/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens and *M. gallisepticum* P1C5/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens (Supplemental Tables 8 and 9).

Notable upregulated pathways between *M. gallisepticum* P1C5/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens (Supplemental Table 9) and Mock/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens include the toll-like receptor, phagosome, metabolic, apoptosis, RIG-I-like receptor signaling, focal adhesion, and ECM-receptor interaction pathways (Supplemental Table 8).

The influenza A response pathway is significantly affected across multiple comparisons. This pathway is significantly upregulated between *M. gallisepticum*

P1C5/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens (SupplementalTable 9) and Mock/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens (Supplemental Table 8) and is downregulated between Mock/H3N8 and *M. gallisepticum* P1C5/H3N8 infected chickens (Supplemental Table 7). Regulation of the actin cytoskeleton is also significantly upregulated between *M. gallisepticum* P1C5/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens (Supplemental Table 9) and Mock/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens (Supplemental Table 8).

Discussion

These data presented contribute to our further understanding of the co-pathogenesis of *Mycoplasma gallisepticum* and LPAIV. the exacerbated disease phenotype produced by these co-pathogens has been described using an *ex vivo* model system and *in vivo* at a later time point of 15 days post-infection with alternate strains of *M. gallisepticum* and LPAIV [7,8,9]. In our *in* vivo model examining this co-pathogenesis at an early timepoint, tracheal histopathology was significantly more severe in *M. gallisepticum* R_{low}/H3N8 co-infected chickens compared to Mock/H3N8 mono-infected chickens, both infected with H3N8 for a total of 3 days (Figure 1A and 1B).

In contrast, the inverse order of infection yielded no significant differences in tracheal histopathology between H3N8/*M. gallisepticum* R_{low} co-infected chickens and Mock/*M. gallisepticum* R_{low} mono-infected chickens both receiving *M. gallisepticum* R_{low} infection for 3 days (Figure 1A and 1B). Although we could not discern exacerbation of pathologic lesions due to co-pathogenesis between these groups, the recovery of live *M. gallisepticum* R_{low} from the tracheas of H3N8/*M. gallisepticum* R_{low} co-infected

chickens was notably higher than in Mock/*M. gallisepticum* R_{low} mono-infected chickens (Figure 2A). Previous *in vivo* studies by Stipkovits *et al*. yielded significant differences between mono- and co-infected chickens in a similar model of *M. gallisepticum* and H3N8 co-pathogenesis, however our model examines co-infection at a more acute stage, 6 days post-infection, as seen in experiment #1 [7, 8]. The increased persistence of *M. gallisepticum* R_{low} in H3N8/*M. gallisepticum* R_{low} co-infected chickens at 6 days post-infection may contribute to more dramatic differences in tracheal pathology between mono- and co-infected birds over a longer period.

Although previous works have utilized the transcriptomic approach to monitor *M. gallisepticum* infection of chickens, our work furthers the use of these techniques in an *M. gallisepticum* co-infection experiment *in vivo* [3,18,22,23]. This method of capturing the global response to infection in the airway highlighted a number of critical host responses during this co-infection model. One of these responses was the significant downregulation of pathways contributing to ciliary activity and integrity in the trachea. Although prior works have examined this *ex vivo*, the data presented here provides evidence of deleterious impact on the ciliary mucosa function in the natural chicken host at a transcript level [9]. This reduction of ciliary activity was shown between *M. gallisepticum* P1C5/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens as well as Mock/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens, indicating that there is a unique relationship between co-infection with a virulent *M. gallisepticum* and LPAIV over an attenuated *M. gallisepticum*, such as the mutant P1C5, or infection with LPAIV alone. Loss of the integrity of the mucociliary elevator could heavily contribute to the

exacerbated co-pathogenesis by inhibiting pathogen clearing and promoting the inflammatory immune response due to damage in the tracheal mucosa.

Another novel aspect of these experiments is the differential expression of TLR genes in response to mono- and co-infection with *M. gallisepticum* and LPAIV. TLR15, which is significantly decreased in expression in Mock*/M. gallisepticum* R_{low} infected chickens compared to Mock/H3N8 infected chickens (Table 1), has also been shown to be abundantly expressed in response to *M. gallisepticum* R_{low} infection in chickens [3]. Our current findings suggest that modulation in the expression of TLR15 remains critical in response to *M. gallisepticum*, however it is more highly expressed in response to LPAIV H3N8 alone. This could indicate that a TLR15-mediated response is more relevant in response to influenza virus mono-infection and is not enhanced in the presence of co-infection with *M. gallisepticum*.

TLR21 expression is suppressed during co-infection, in that between both Mock/H3N8 and *M. gallisepticum* co-infection, TLR21 was significantly decreased in expression (Table 1). This suppression of TLR21 during *M.* gallisepticum co-infection negatively correlates with the significantly enhanced tracheal pathology and viral loads in co-infected chickens (Figure 1A, 1B, and 2B). In ducks, TLR21 is upregulated in the blood, spleen, bursa, and cecum in response to duck plague virus (DPV) and serves to activate NFκB and the transcription of IL-6, IFNα, and IL-1β [24].

In chicken bone marrow macrophages, TLR21 and TLR4 co-stimulation induces increases in pro-inflammatory genes such as IL-1β, IL-12p40, and IL-10 and nitric oxide production [25]. Therefore, suppression of TLR21 signaling in response to CpG deoxynucleotides and potential disruption of the association with TLR4 during co-

infection with *Mycoplasma gallisepticum* may also be a critical factor in our model of co-pathogenesis.

Increased expression of IL-1β was also documented between *M. gallisepticum* R_{low}/H3N8 and *M. gallisepticum* P1C5/H3N8 infected experimental groups, indicating a possible differential effect on activation of the NLRP3 inflammasome by IL-1β during co-infection. Two MMP genes, MMP1 and MMP9, were also differentially expressed among experimental groups. MMP1, was differentially expressed between Mock/H3N8 and Mock/*M gallisepticum* R_{low} infected groups in experiment #1. These data can be associated with the increased tracheal pathology seen in the Mock/*M. gallisepticum* R_{low} group over the Mock/H3N8 group, although this increase is not statistically significant (Figure 1A and 1B). MMP7 was among the highest expressed genes in the trachea in response to *M. gallisepticum* R_{low} infection of chickens [3]. MMP1 and MMP7 have both been implicated as biomarkers of human idiopathic pulmonary fibrosis and are overexpressed in the lungs of patients with chronic pulmonary disease [26]. Therefore, a precedent exists for these MMP genes being involved in tissue remodeling in response to ongoing inflammatory challenge such as that induced by *M. gallisepticum* infection and potential association with the manifestation of pathologic lesions in the airway.

Functional pathway analysis also determined the differential regulation of the response to influenza A virus pathway among comparisons between mono- and co-infected chickens illustrates the importance of modulation of this specific response mechanism. Co-infection with virulent *M. gallisepticum* appears to have a unique effect on this response, as the pathway was upregulated *M. gallisepticum* P1C5/H3N8 and *M.*

gallisepticum R_{low}/H3N8 infected chickens and Mock/H3N8 and *M. gallisepticum* R_{low}/H3N8 infected chickens and downregulated between Mock/H3N8 and *M. gallisepticum* P1C5/H3N8 infected chickens. Although this influenza A response pathway is increased in expression, it may not yield a productive immune response to clear the virus during co-infection, as evidenced by the highest tracheal viral load being present in the *M. gallisepticum* R_{low}/H3N8 infected group of experiment #1 (Figure 2B).

A final novel finding of these experiments was the persistence of attenuated *M. gallisepticum* mutants P1C5 6 days post-infection and P1H9 7 days post-infection in the chicken trachea in experiments #2 and #3. Previously, these mutants have been shown to be completely cleared from the birds at 14 days post-infection [11,17]. The ability of these attenuated mutants to persist in the shorter term illustrates that the absence of a functional neuraminidase gene and the Mycoplasma specific lipoprotein A (MslA) in *M. gallisepticum* do not prohibit early colonization and replication in the chicken trachea. The *M. gallisepticum* MslA mutant P1H9 was only able to persist to a chronic state at 14 days post-infection when in the presence of H3N8 co-infection. Although the *M. gallisepticum* mutant P1C5 did persist at low titers 14 days post-infection in experiment #3, this survival did not appear to be impacted by co-infection with H3N8. This contrast to previous findings on the chronic persistence of P1C5 *in vivo* could be attributed to experimental variation.

Co-infection with H3N8 likely induces changes in the host environment, such as alterations in the immune response and changes in the tracheal architecture that partially rescue the mutant phenotype to allow for the its persistence. The capacity of LPAIV to rescue persistence of an attenuated mutant to levels of its parent strain

indicate that co-infection in populations of chickens infected with a sub-clinical *M. gallisepticum* field isolate are still susceptible to potential deleterious effects of co-pathogenesis. Furthermore, these findings have profound implications for potential consequences of LPAIV outbreaks in flocks recently immunized with live, attenuated *M. gallisepticum* vaccines (*e.g.*, TS-11, 6/85).

Overall, our findings contribute to the understanding of what underlies the co-pathogenesis of *M. gallisepticum* and LPAIV in chickens. This co-pathogen relationship does not appear to be neuraminidase dependent as previously hypothesized. In addition to impacting commercial flocks, these findings are relevant to backyard free range flocks exposed to a primary infection with either *M. gallisepticum* or LPAIV isolates of various virulence that may evade common clinical surveillance measures, and could result in severe economic losses to farmers and increased loads of the secondary pathogen if not properly addressed. Overall, our findings contribute to the understanding of host-microbe interactions during the co-pathogenesis of *M. gallisepticum* and LPAIV in chickens and indicate that the co-pathogenesis of *M. gallisepticum* and LPAIV is a complex dynamic that warrants further experimental analysis.

Acknowledgements

The authors thank Dr. Kirklyn Kerr for his expertise in veterinary pathology and assistance during necropsy and Katherine Pflaum, Arlind Mara, and Tyler Gavitt for assistance during the chicken experiments and for helpful experimental discussions. We also thank the Connecticut Veterinary Medical Diagnostic Laboratory for assistance in AIV testing. Funding for these experiments was generously provided by the Center of

Excellence for Vaccine Research (CEVR) and the United States Department of Agriculture (USDA) Hatch grant program.

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Supplemental Data

Supplemental Table 1

Mock/H3N8 vs. R(low)/H3N8 Downregulated DEG DAVID Gene Ontology				
Term	**Count**	**%**	**P-Value**	**Benjamini**
cilium assembly	17	5.9	1.10E-13	4.90E-11
intraciliary transport	4	1.4	3.30E-04	6.90E-02
microtubule-based movement	6	2.1	6.20E-04	8.60E-02
cilium movement	4	1.4	1.10E-03	1.10E-01
neural tube patterning	3	1	1.50E-03	1.30E-01
nonmotile primary cilium assembly	4	1.4	3.40E-03	2.20E-01
intraciliary retrograde transport	3	1	3.80E-03	2.10E-01
epithelial cilium movement	3	1	5.20E-03	2.50E-01
inner dynein arm assembly	3	1	6.90E-03	2.80E-01
motile cilium assembly	3	1	6.90E-03	2.80E-01
sperm motility	4	1.4	8.30E-03	3.00E-01
cilium morphogenesis	4	1.4	1.00E-02	3.30E-01
fat cell differentiation	4	1.4	2.80E-02	6.50E-01
smoothened signaling pathway	4	1.4	3.40E-02	6.90E-01
multi-ciliated epithelial cell differentiation	2	0.7	4.80E-02	7.80E-01
cilium movement involved in cell motility	2	0.7	4.80E-02	7.80E-01
prostaglandin metabolic process	2	0.7	4.80E-02	7.80E-01
epithelial cilium movement involved in determination of left/right asymmetry	2	0.7	6.30E-02	8.50E-01
retina development in camera-type eye	3	1	7.10E-02	8.60E-01
determination of left/right symmetry	3	1	9.00E-02	9.10E-01
UTP biosynthetic process	2	0.7	1.20E-01	9.60E-01
CTP biosynthetic process	2	0.7	1.20E-01	9.60E-01
GTP biosynthetic process	2	0.7	1.20E-01	9.60E-01
ventricular system development	2	0.7	1.50E-01	9.80E-01
intracellular transport	2	0.7	1.50E-01	9.80E-01
protein localization to cilium	2	0.7	1.60E-01	9.80E-01
glycogen metabolic process	2	0.7	1.60E-01	9.80E-01
transcription from RNA polymerase III promoter	2	0.7	1.80E-01	9.80E-01
transcription from RNA polymerase I promoter	2	0.7	1.80E-01	9.80E-01
muscle cell differentiation	2	0.7	1.90E-01	9.90E-01
multicellular organism growth	3	1	2.00E-01	9.90E-01
small GTPase mediated signal transduction	5	1.7	2.40E-01	9.90E-01
positive regulation of macroautophagy	2	0.7	2.70E-01	1.00E+00
glycolytic process	2	0.7	2.80E-01	1.00E+00
tricarboxylic acid cycle	2	0.7	2.80E-01	1.00E+00
mitotic spindle assembly	2	0.7	3.00E-01	1.00E+00
lens development in camera-type eye	2	0.7	3.10E-01	1.00E+00
dorsal/ventral pattern formation	2	0.7	3.10E-01	1.00E+00

skeletal system morphogenesis	2	0.7	3.30E-01	1.00E+00
cytoplasmic microtubule organization	2	0.7	3.60E-01	1.00E+00
protein folding	3	1	3.60E-01	1.00E+00
homophilic cell adhesion via plasma membrane adhesion molecules	3	1	3.90E-01	1.00E+00
defense response to bacterium	2	0.7	4.20E-01	1.00E+00
cartilage development	2	0.7	4.30E-01	1.00E+00
heart looping	2	0.7	4.30E-01	1.00E+00
spermatid development	2	0.7	4.30E-01	1.00E+00
protein autophosphorylation	3	1	4.40E-01	1.00E+00
hematopoietic progenitor cell differentiation	2	0.7	4.70E-01	1.00E+00
heart development	3	1	4.70E-01	1.00E+00
male gonad development	2	0.7	4.80E-01	1.00E+00
kidney development	2	0.7	5.00E-01	1.00E+00
embryonic digit morphogenesis	2	0.7	5.10E-01	1.00E+00
positive regulation of sequence-specific DNA binding transcription factor activity	2	0.7	5.10E-01	1.00E+00
transforming growth factor beta receptor signaling pathway	2	0.7	5.20E-01	1.00E+00
Golgi organization	2	0.7	5.70E-01	1.00E+00
palate development	2	0.7	5.90E-01	1.00E+00
vesicle-mediated transport	2	0.7	6.10E-01	1.00E+00
brain development	2	0.7	6.30E-01	1.00E+00
intracellular signal transduction	4	1.4	6.60E-01	1.00E+00
mitophagy in response to mitochondrial depolarization	2	0.7	6.90E-01	1.00E+00
negative regulation of cell proliferation	3	1	7.00E-01	1.00E+00
visual perception	2	0.7	7.20E-01	1.00E+00
spermatogenesis	2	0.7	7.30E-01	1.00E+00
carbohydrate metabolic process	2	0.7	7.30E-01	1.00E+00
protein phosphorylation	2	0.7	7.40E-01	1.00E+00
cell migration	2	0.7	7.80E-01	1.00E+00
transcription, DNA-templated	6	2.1	7.90E-01	1.00E+00
transcription from RNA polymerase II promoter	2	0.7	7.90E-01	1.00E+00
regulation of transcription from RNA polymerase II promoter	3	1	8.10E-01	1.00E+00
positive regulation of transcription, DNA-templated	3	1	8.20E-01	1.00E+00
inflammatory response	2	0.7	8.70E-01	1.00E+00
cell adhesion	2	0.7	8.80E-01	1.00E+00
positive regulation of transcription from RNA polymerase II promoter	5	1.7	8.90E-01	1.00E+00
regulation of transcription, DNA-templated	4	1.4	9.40E-01	1.00E+00

Supplemental Table 2

Mock/H3N8 vs. R(low)/H3N8 Upregulated DEG DAVID Gene Ontology				
Term	**Count**	**%**	**P-Value**	**Benjamini**
immune response	16	6.2	8.80E-09	7.50E-06

B cell receptor signaling pathway	9	3.5	9.20E-09	3.90E-06
inflammatory response	15	5.8	3.50E-07	1.00E-04
regulation of cell proliferation	13	5	2.20E-06	4.60E-04
transmembrane receptor protein tyrosine kinase signaling pathway	9	3.5	2.90E-05	4.80E-03
T cell receptor signaling pathway	6	2.3	1.90E-04	2.70E-02
innate immune response	10	3.8	3.20E-04	3.80E-02
apoptotic cell clearance	4	1.5	1.00E-03	1.00E-01
antigen processing and presentation	5	1.9	1.50E-03	1.30E-01
neutrophil chemotaxis	5	1.9	1.80E-03	1.40E-01
apoptotic signaling pathway	5	1.9	2.40E-03	1.70E-01
germinal center formation	3	1.2	2.60E-03	1.70E-01
endodermal cell differentiation	4	1.5	4.40E-03	2.50E-01
peptidyl-tyrosine autophosphorylation	5	1.9	4.50E-03	2.40E-01
brown fat cell differentiation	4	1.5	5.20E-03	2.60E-01
positive regulation of interleukin-2 biosynthetic process	3	1.2	8.90E-03	3.80E-01
extracellular matrix organization	6	2.3	9.50E-03	3.80E-01
I-kappaB kinase/NF-kappaB signaling	4	1.5	9.60E-03	3.60E-01
positive regulation of apoptotic process	7	2.7	1.40E-02	4.80E-01
negative thymic T cell selection	3	1.2	1.50E-02	4.70E-01
proteolysis involved in cellular protein catabolic process	4	1.5	1.60E-02	4.70E-01
regulation of apoptotic process	7	2.7	1.60E-02	4.60E-01
positive regulation of B cell proliferation	4	1.5	1.90E-02	5.10E-01
response to lipopolysaccharide	5	1.9	2.60E-02	6.10E-01
defense response to virus	5	1.9	2.60E-02	6.10E-01
cell differentiation	8	3.1	3.60E-02	7.10E-01
positive regulation of I-kappaB kinase/NF-kappaB signaling	6	2.3	3.80E-02	7.20E-01
intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress	3	1.2	4.00E-02	7.20E-01
granuloma formation	2	0.8	4.20E-02	7.30E-01
cyclooxygenase pathway	2	0.8	4.20E-02	7.30E-01
positive regulation of interferon-gamma production	3	1.2	5.00E-02	7.80E-01
chemokine-mediated signaling pathway	3	1.2	5.50E-02	8.00E-01
antigen processing and presentation of endogenous peptide antigen via MHC class I via ER pathway, TAP-dependent	2	0.8	6.30E-02	8.30E-01
antigen processing and presentation of endogenous peptide antigen via MHC class Ib via ER pathway, TAP-dependent	2	0.8	6.30E-02	8.30E-01
positive regulation of platelet activation	2	0.8	6.30E-02	8.30E-01
immunological synapse formation	2	0.8	6.30E-02	8.30E-01
positive regulation of antigen processing and presentation of peptide antigen via MHC class I	2	0.8	6.30E-02	8.30E-01
positive regulation of tumor necrosis factor biosynthetic process	2	0.8	6.30E-02	8.30E-01
positive regulation of protein kinase B signaling	4	1.5	6.30E-02	8.20E-01
positive regulation of protein kinase activity	3	1.2	6.70E-02	8.30E-01
integrin-mediated signaling pathway	4	1.5	6.70E-02	8.20E-01
toll-like receptor signaling pathway	3	1.2	7.30E-02	8.40E-01
negative regulation of leukocyte apoptotic process	2	0.8	8.30E-02	8.70E-01

cotranslational protein targeting to membrane	2	0.8	8.30E-02	8.70E-01
positive regulation of MHC class II biosynthetic process	2	0.8	8.30E-02	8.70E-01
dsRNA transport	2	0.8	8.30E-02	8.70E-01
antigen processing and presentation of exogenous protein antigen via MHC class Ib, TAP-dependent	2	0.8	8.30E-02	8.70E-01
protein N-linked glycosylation via asparagine	3	1.2	8.60E-02	8.70E-01
intrinsic apoptotic signaling pathway in response to DNA damage	3	1.2	8.60E-02	8.70E-01
chemotaxis	3	1.2	9.90E-02	9.00E-01
positive regulation of alpha-beta T cell proliferation	2	0.8	1.00E-01	9.00E-01
positive regulation of keratinocyte migration	2	0.8	1.00E-01	9.00E-01
toll-like receptor 3 signaling pathway	2	0.8	1.00E-01	9.00E-01
negative regulation of interleukin-17 production	2	0.8	1.00E-01	9.00E-01
secretion by cell	2	0.8	1.00E-01	9.00E-01
positive regulation of NF-kappaB transcription factor activity	4	1.5	1.00E-01	9.00E-01
positive regulation of MAP kinase activity	3	1.2	1.10E-01	9.00E-01
response to endoplasmic reticulum stress	3	1.2	1.10E-01	9.10E-01
cellular response to amino acid stimulus	3	1.2	1.20E-01	9.20E-01
complement activation, classical pathway	2	0.8	1.20E-01	9.20E-01
positive thymic T cell selection	2	0.8	1.20E-01	9.20E-01
regulation of cytokine secretion	2	0.8	1.20E-01	9.20E-01
negative regulation of protein phosphorylation	3	1.2	1.30E-01	9.20E-01
regulation of blood pressure	3	1.2	1.30E-01	9.20E-01
negative regulation of apoptotic process	7	2.7	1.30E-01	9.20E-01
activation of cysteine-type endopeptidase activity involved in apoptotic process	3	1.2	1.30E-01	9.20E-01
cell migration	5	1.9	1.40E-01	9.30E-01
immunoglobulin mediated immune response	2	0.8	1.40E-01	9.30E-01
activation of MAPKKK activity	2	0.8	1.40E-01	9.30E-01
regulation of osteoclast differentiation	2	0.8	1.40E-01	9.30E-01
adaptive immune response	3	1.2	1.40E-01	9.20E-01
regulation of protein localization	3	1.2	1.40E-01	9.20E-01
negative regulation of sequence-specific DNA binding transcription factor activity	3	1.2	1.50E-01	9.30E-01
positive regulation of GTPase activity	4	1.5	1.50E-01	9.30E-01
ossification	3	1.2	1.60E-01	9.30E-01
intracellular signal transduction	8	3.1	1.60E-01	9.30E-01
activation of NF-kappaB-inducing kinase activity	2	0.8	1.60E-01	9.30E-01
B cell activation	2	0.8	1.60E-01	9.30E-01
regulation of immune response	2	0.8	1.60E-01	9.30E-01
chaperone mediated protein folding requiring cofactor	2	0.8	1.60E-01	9.30E-01
negative regulation of B cell proliferation	2	0.8	1.60E-01	9.30E-01
positive regulation of calcium-mediated signaling	2	0.8	1.60E-01	9.30E-01
negative regulation of smoothened signaling pathway	2	0.8	1.60E-01	9.30E-01
myeloid dendritic cell differentiation	2	0.8	1.60E-01	9.30E-01
negative regulation of interferon-gamma production	2	0.8	1.60E-01	9.30E-01

defense response to protozoan	2	0.8	1.60E-01	9.30E-01
T cell costimulation	2	0.8	1.60E-01	9.30E-01
release of cytochrome c from mitochondria	2	0.8	1.60E-01	9.30E-01
collagen catabolic process	2	0.8	1.60E-01	9.30E-01
positive regulation of MAPK cascade	3	1.2	1.60E-01	9.30E-01
positive regulation of JNK cascade	3	1.2	1.60E-01	9.30E-01
positive regulation of protein phosphorylation	3	1.2	1.70E-01	9.40E-01
superoxide anion generation	2	0.8	1.80E-01	9.40E-01
positive regulation of transcription factor import into nucleus	2	0.8	1.80E-01	9.40E-01
positive regulation of interleukin-4 production	2	0.8	1.80E-01	9.40E-01
positive regulation of B cell differentiation	2	0.8	1.80E-01	9.40E-01
cellular response to lipopolysaccharide	3	1.2	1.90E-01	9.50E-01
positive regulation of pri-miRNA transcription from RNA polymerase II promoter	2	0.8	1.90E-01	9.50E-01
lymphocyte chemotaxis	2	0.8	1.90E-01	9.50E-01
positive regulation of receptor-mediated endocytosis	2	0.8	1.90E-01	9.50E-01
positive regulation of peptidyl-tyrosine phosphorylation	3	1.2	2.10E-01	9.60E-01
cell-matrix adhesion	3	1.2	2.10E-01	9.60E-01
negative regulation of interleukin-6 production	2	0.8	2.10E-01	9.60E-01
positive regulation of release of cytochrome c from mitochondria	2	0.8	2.10E-01	9.60E-01
G-protein coupled receptor signaling pathway	5	1.9	2.20E-01	9.70E-01
ER to Golgi vesicle-mediated transport	3	1.2	2.30E-01	9.70E-01
extrinsic apoptotic signaling pathway in absence of ligand	2	0.8	2.30E-01	9.70E-01
actin filament polymerization	2	0.8	2.30E-01	9.70E-01
positive regulation of phosphatidylinositol 3-kinase signaling	3	1.2	2.30E-01	9.70E-01
neural crest cell migration	3	1.2	2.30E-01	9.70E-01
complement activation	2	0.8	2.40E-01	9.70E-01
MyD88-dependent toll-like receptor signaling pathway	2	0.8	2.40E-01	9.70E-01
regulation of G-protein coupled receptor protein signaling pathway	2	0.8	2.40E-01	9.70E-01
positive regulation of phagocytosis	2	0.8	2.60E-01	9.80E-01
positive regulation of vascular endothelial growth factor production	2	0.8	2.80E-01	9.80E-01
positive regulation of DNA binding	2	0.8	2.80E-01	9.80E-01
negative regulation of tumor necrosis factor production	2	0.8	2.80E-01	9.80E-01
protein kinase B signaling	2	0.8	2.80E-01	9.80E-01
endoplasmic reticulum organization	2	0.8	2.80E-01	9.80E-01
cell redox homeostasis	3	1.2	2.80E-01	9.80E-01
response to oxidative stress	3	1.2	2.90E-01	9.80E-01
positive regulation of gene expression	5	1.9	2.90E-01	9.80E-01
oligosaccharide metabolic process	2	0.8	2.90E-01	9.80E-01
monocyte chemotaxis	2	0.8	2.90E-01	9.80E-01
negative regulation of protein catabolic process	2	0.8	3.10E-01	9.90E-01
positive regulation of inflammatory response	2	0.8	3.10E-01	9.90E-01
angiogenesis	4	1.5	3.10E-01	9.90E-01
phosphorylation	2	0.8	3.20E-01	9.90E-01
signal transduction	7	2.7	3.20E-01	9.90E-01

transcription from RNA polymerase II promoter	4	1.5	3.30E-01	9.90E-01
phosphatidylinositol dephosphorylation	2	0.8	3.40E-01	9.90E-01
growth	2	0.8	3.40E-01	9.90E-01
cell maturation	2	0.8	3.40E-01	9.90E-01
cellular response to interferon-gamma	2	0.8	3.40E-01	9.90E-01
glucose homeostasis	3	1.2	3.50E-01	9.90E-01
regulation of transcription from RNA polymerase II promoter	6	2.3	3.60E-01	9.90E-01
regulation of cell adhesion	2	0.8	3.60E-01	9.90E-01
protein homooligomerization	4	1.5	3.70E-01	9.90E-01
cellular response to drug	2	0.8	3.80E-01	9.90E-01
mitotic spindle assembly	2	0.8	3.80E-01	9.90E-01
platelet aggregation	2	0.8	3.90E-01	9.90E-01
erythrocyte differentiation	2	0.8	4.00E-01	9.90E-01
regulation of cell shape	3	1.2	4.10E-01	9.90E-01
negative regulation of gene expression	3	1.2	4.10E-01	9.90E-01
protein stabilization	3	1.2	4.10E-01	9.90E-01
cellular response to mechanical stimulus	2	0.8	4.20E-01	9.90E-01
defense response to Gram-negative bacterium	2	0.8	4.20E-01	9.90E-01
cellular response to insulin stimulus	2	0.8	4.30E-01	1.00E+00
calcium ion transport	2	0.8	4.40E-01	1.00E+00
histone H3 acetylation	2	0.8	4.50E-01	1.00E+00
cellular response to interleukin-1	2	0.8	4.50E-01	1.00E+00
positive regulation of fat cell differentiation	2	0.8	4.50E-01	1.00E+00
cell division	3	1.2	4.60E-01	1.00E+00
cell chemotaxis	2	0.8	4.70E-01	1.00E+00
protein homotetramerization	2	0.8	4.70E-01	1.00E+00
regulation of inflammatory response	2	0.8	4.80E-01	1.00E+00
response to virus	2	0.8	4.80E-01	1.00E+00
protein folding	3	1.2	5.00E-01	1.00E+00
chloride transmembrane transport	2	0.8	5.10E-01	1.00E+00
defense response to bacterium	2	0.8	5.10E-01	1.00E+00
protein import into nucleus	2	0.8	5.10E-01	1.00E+00
cellular response to hypoxia	2	0.8	5.10E-01	1.00E+00
B cell differentiation	2	0.8	5.20E-01	1.00E+00
cell adhesion	4	1.5	5.20E-01	1.00E+00
cellular response to tumor necrosis factor	2	0.8	5.30E-01	1.00E+00
positive regulation of ERK1 and ERK2 cascade	3	1.2	5.40E-01	1.00E+00
activation of MAPK activity	2	0.8	5.50E-01	1.00E+00
positive regulation of protein binding	2	0.8	5.60E-01	1.00E+00
skeletal system development	2	0.8	5.70E-01	1.00E+00
fat cell differentiation	2	0.8	5.90E-01	1.00E+00
negative regulation of protein kinase activity	2	0.8	6.20E-01	1.00E+00
cell surface receptor signaling pathway	3	1.2	6.30E-01	1.00E+00
regulation of cell cycle	2	0.8	6.30E-01	1.00E+00

cellular calcium ion homeostasis	2	0.8	6.50E-01	1.00E+00
positive regulation of angiogenesis	2	0.8	6.50E-01	1.00E+00
positive regulation of cell proliferation	4	1.5	6.50E-01	1.00E+00
protein glycosylation	2	0.8	6.80E-01	1.00E+00
cytokine-mediated signaling pathway	2	0.8	6.80E-01	1.00E+00
response to hypoxia	2	0.8	6.80E-01	1.00E+00
cell proliferation	3	1.2	6.90E-01	1.00E+00
osteoblast differentiation	2	0.8	7.00E-01	1.00E+00
transport	2	0.8	7.20E-01	1.00E+00
transmembrane transport	2	0.8	7.20E-01	1.00E+00
negative regulation of cell growth	2	0.8	7.30E-01	1.00E+00
brain development	2	0.8	7.30E-01	1.00E+00
cytoskeleton organization	2	0.8	7.40E-01	1.00E+00
positive regulation of transcription from RNA polymerase II promoter	8	3.1	7.50E-01	1.00E+00
negative regulation of neuron apoptotic process	2	0.8	7.50E-01	1.00E+00
transcription, DNA-templated	8	3.1	7.60E-01	1.00E+00
protein transport	2	0.8	7.80E-01	1.00E+00
proteolysis	2	0.8	7.80E-01	1.00E+00
spermatogenesis	2	0.8	8.20E-01	1.00E+00
carbohydrate metabolic process	2	0.8	8.20E-01	1.00E+00
negative regulation of cell proliferation	3	1.2	8.30E-01	1.00E+00
small GTPase mediated signal transduction	3	1.2	8.40E-01	1.00E+00
apoptotic process	2	0.8	8.50E-01	1.00E+00
protein autophosphorylation	2	0.8	8.60E-01	1.00E+00
positive regulation of cell migration	2	0.8	8.80E-01	1.00E+00
oxidation-reduction process	2	0.8	8.90E-01	1.00E+00
protein ubiquitination	2	0.8	8.90E-01	1.00E+00
multicellular organism development	3	1.2	9.00E-01	1.00E+00
positive regulation of transcription, DNA-templated	3	1.2	9.20E-01	1.00E+00
regulation of transcription, DNA-templated	5	1.9	9.60E-01	1.00E+00
negative regulation of transcription, DNA-templated	2	0.8	9.70E-01	1.00E+00
negative regulation of transcription from RNA polymerase II promoter	3	1.2	9.80E-01	1.00E+00

Supplemental Table 3

P1C5/H3N8 vs. R(low)/H3N8 Upregulated DEG DAVID Gene Ontology				
Term	**Count**	**%**	**P-Value**	**Benjamini**
inflammatory response	15	5.9	1.80E-07	1.70E-04
immune response	14	5.5	2.70E-07	1.20E-04
regulation of cell proliferation	12	4.7	8.20E-06	2.50E-03
innate immune response	10	4	2.10E-04	4.70E-02
response to lipopolysaccharide	7	2.8	6.60E-04	1.10E-01
toll-like receptor signaling pathway	5	2	7.40E-04	1.10E-01
germinal center formation	3	1.2	2.40E-03	2.70E-01

endodermal cell differentiation	4	1.6	3.80E-03	3.50E-01
cellular response to hypoxia	5	2	4.20E-03	3.40E-01
defense response to virus	6	2.4	4.20E-03	3.20E-01
brown fat cell differentiation	4	1.6	4.50E-03	3.10E-01
regulation of cytokine secretion	3	1.2	5.80E-03	3.50E-01
transmembrane receptor protein tyrosine kinase signaling pathway	6	2.4	6.20E-03	3.50E-01
cell differentiation	9	3.6	9.10E-03	4.50E-01
positive regulation of apoptotic process	7	2.8	1.10E-02	5.00E-01
integrin-mediated signaling pathway	5	2	1.20E-02	4.90E-01
neutrophil chemotaxis	4	1.6	1.40E-02	5.20E-01
apoptotic cell clearance	3	1.2	1.60E-02	5.70E-01
positive regulation of pri-miRNA transcription from RNA polymerase II promoter	3	1.2	1.60E-02	5.70E-01
apoptotic signaling pathway	4	1.6	1.70E-02	5.50E-01
negative regulation of smooth muscle cell proliferation	3	1.2	2.00E-02	6.00E-01
peptide cross-linking	3	1.2	2.40E-02	6.40E-01
peptidyl-tyrosine autophosphorylation	4	1.6	2.60E-02	6.70E-01
MyD88-dependent toll-like receptor signaling pathway	3	1.2	2.70E-02	6.70E-01
positive regulation of axon extension	3	1.2	3.20E-02	7.00E-01
positive regulation of smooth muscle cell proliferation	3	1.2	3.60E-02	7.30E-01
protein kinase B signaling	3	1.2	3.60E-02	7.30E-01
cell migration	6	2.4	4.00E-02	7.60E-01
cyclooxygenase pathway	2	0.8	4.00E-02	7.50E-01
negative regulation of regulatory T cell differentiation	2	0.8	4.00E-02	7.50E-01
heme catabolic process	2	0.8	4.00E-02	7.50E-01
hypermethylation of CpG island	2	0.8	4.00E-02	7.50E-01
tolerance induction to lipopolysaccharide	2	0.8	4.00E-02	7.50E-01
granuloma formation	2	0.8	4.00E-02	7.50E-01
positive regulation of inflammatory response	3	1.2	4.50E-02	7.80E-01
positive regulation of macroautophagy	3	1.2	5.60E-02	8.30E-01
regulation of innate immune response	2	0.8	5.90E-02	8.40E-01
positive regulation of platelet activation	2	0.8	5.90E-02	8.40E-01
positive regulation of tumor necrosis factor biosynthetic process	2	0.8	5.90E-02	8.40E-01
B-1 B cell homeostasis	2	0.8	5.90E-02	8.40E-01
positive regulation of interleukin-12 biosynthetic process	2	0.8	5.90E-02	8.40E-01
negative regulation of B cell activation	2	0.8	5.90E-02	8.40E-01
I-kappaB kinase/NF-kappaB signaling	3	1.2	6.70E-02	8.70E-01
B cell receptor signaling pathway	3	1.2	7.20E-02	8.80E-01
platelet aggregation	3	1.2	7.80E-02	8.90E-01
secretion	2	0.8	7.90E-02	8.90E-01
detection of lipopolysaccharide	2	0.8	7.90E-02	8.90E-01
response to oxidative stress	4	1.6	8.00E-02	8.80E-01
antigen processing and presentation	3	1.2	8.40E-02	8.90E-01
cellular response to mechanical stimulus	3	1.2	9.00E-02	9.00E-01
proteolysis involved in cellular protein catabolic process	3	1.2	9.00E-02	9.00E-01

positive regulation of MAP kinase activity	3	1.2	9.60E-02	9.10E-01
secretion by cell	2	0.8	9.70E-02	9.10E-01
extracellular matrix disassembly	2	0.8	9.70E-02	9.10E-01
endoderm formation	2	0.8	9.70E-02	9.10E-01
negative regulation of interleukin-17 production	2	0.8	9.70E-02	9.10E-01
positive regulation of keratinocyte migration	2	0.8	9.70E-02	9.10E-01
positive regulation of I-kappaB kinase/NF-kappaB signaling	5	2	9.90E-02	9.10E-01
substrate adhesion-dependent cell spreading	3	1.2	1.00E-01	9.10E-01
negative regulation of apoptotic process	7	2.8	1.10E-01	9.20E-01
cellular response to amino acid stimulus	3	1.2	1.10E-01	9.10E-01
natural killer cell differentiation	2	0.8	1.20E-01	9.20E-01
cellular response to interferon-beta	2	0.8	1.20E-01	9.20E-01
negative regulation of interleukin-2 production	2	0.8	1.20E-01	9.20E-01
positive regulation of receptor biosynthetic process	2	0.8	1.20E-01	9.20E-01
regulation of blood pressure	3	1.2	1.20E-01	9.20E-01
positive regulation of gene expression	6	2.4	1.20E-01	9.10E-01
response to virus	3	1.2	1.20E-01	9.20E-01
regulation of inflammatory response	3	1.2	1.20E-01	9.20E-01
regulation of apoptotic process	5	2	1.30E-01	9.20E-01
regulation of I-kappaB kinase/NF-kappaB signaling	2	0.8	1.30E-01	9.30E-01
regulation of cell adhesion mediated by integrin	2	0.8	1.30E-01	9.30E-01
positive regulation of interleukin-2 biosynthetic process	2	0.8	1.30E-01	9.30E-01
DNA methylation involved in gamete generation	2	0.8	1.30E-01	9.30E-01
regulation of osteoclast differentiation	2	0.8	1.30E-01	9.30E-01
fatty acid transport	2	0.8	1.30E-01	9.30E-01
positive regulation of macrophage cytokine production	2	0.8	1.30E-01	9.30E-01
negative regulation of sequence-specific DNA binding transcription factor activity	3	1.2	1.40E-01	9.30E-01
ossification	3	1.2	1.40E-01	9.40E-01
positive regulation of MAPK cascade	3	1.2	1.50E-01	9.40E-01
release of cytochrome c from mitochondria	2	0.8	1.50E-01	9.40E-01
negative regulation of interferon-gamma production	2	0.8	1.50E-01	9.40E-01
myeloid dendritic cell differentiation	2	0.8	1.50E-01	9.40E-01
defense response to protozoan	2	0.8	1.50E-01	9.40E-01
chaperone mediated protein folding requiring cofactor	2	0.8	1.50E-01	9.40E-01
collagen catabolic process	2	0.8	1.50E-01	9.40E-01
deadenylation-dependent decapping of nuclear-transcribed mRNA	2	0.8	1.50E-01	9.40E-01
protein maturation	2	0.8	1.70E-01	9.50E-01
cell-substrate adhesion	2	0.8	1.70E-01	9.50E-01
signal peptide processing	2	0.8	1.70E-01	9.50E-01
superoxide anion generation	2	0.8	1.70E-01	9.50E-01
astrocyte development	2	0.8	1.80E-01	9.70E-01
positive regulation of phosphorylation	2	0.8	1.80E-01	9.70E-01
G-protein coupled receptor signaling pathway	5	2	2.00E-01	9.70E-01
fat cell differentiation	3	1.2	2.00E-01	9.70E-01

acute-phase response	2	0.8	2.00E-01	9.70E-01
negative regulation of interleukin-6 production	2	0.8	2.00E-01	9.70E-01
positive regulation of release of cytochrome c from mitochondria	2	0.8	2.00E-01	9.70E-01
morphogenesis of an epithelium	2	0.8	2.00E-01	9.70E-01
JAK-STAT cascade	2	0.8	2.00E-01	9.70E-01
cytoplasmic mRNA processing body assembly	2	0.8	2.00E-01	9.70E-01
positive regulation of phosphatidylinositol 3-kinase signaling	3	1.2	2.20E-01	9.80E-01
neural crest cell migration	3	1.2	2.20E-01	9.80E-01
protein targeting to plasma membrane	2	0.8	2.20E-01	9.80E-01
spermatogenesis	4	1.6	2.20E-01	9.70E-01
negative regulation of protein kinase activity	3	1.2	2.30E-01	9.80E-01
regulation of G-protein coupled receptor protein signaling pathway	2	0.8	2.30E-01	9.80E-01
positive regulation of extrinsic apoptotic signaling pathway	2	0.8	2.50E-01	9.80E-01
apoptotic process	4	1.6	2.60E-01	9.80E-01
intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress	2	0.8	2.60E-01	9.90E-01
positive regulation of DNA binding	2	0.8	2.60E-01	9.90E-01
endoplasmic reticulum organization	2	0.8	2.60E-01	9.90E-01
negative regulation of extrinsic apoptotic signaling pathway via death domain receptors	2	0.8	2.60E-01	9.90E-01
negative regulation of tumor necrosis factor production	2	0.8	2.60E-01	9.90E-01
extrinsic apoptotic signaling pathway	2	0.8	2.80E-01	9.90E-01
signal transduction	7	2.8	2.80E-01	9.90E-01
cytokine-mediated signaling pathway	3	1.2	2.90E-01	9.90E-01
negative regulation of endothelial cell apoptotic process	2	0.8	2.90E-01	9.90E-01
positive regulation of interferon-gamma production	2	0.8	2.90E-01	9.90E-01
transcription, DNA-templated	11	4.3	2.90E-01	9.90E-01
chemokine-mediated signaling pathway	2	0.8	3.10E-01	9.90E-01
regulation of mitotic cell cycle	2	0.8	3.10E-01	9.90E-01
osteoblast differentiation	3	1.2	3.10E-01	9.90E-01
cellular iron ion homeostasis	2	0.8	3.20E-01	9.90E-01
cell maturation	2	0.8	3.20E-01	9.90E-01
glucose homeostasis	3	1.2	3.30E-01	9.90E-01
protein homooligomerization	4	1.6	3.40E-01	9.90E-01
one-carbon metabolic process	2	0.8	3.40E-01	9.90E-01
negative regulation of protein ubiquitination	2	0.8	3.40E-01	9.90E-01
regulation of cell adhesion	2	0.8	3.50E-01	9.90E-01
extracellular matrix organization	3	1.2	3.50E-01	9.90E-01
cytoskeleton organization	3	1.2	3.60E-01	9.90E-01
cellular response to drug	2	0.8	3.60E-01	9.90E-01
endosome organization	2	0.8	3.60E-01	9.90E-01
positive regulation of GTPase activity	3	1.2	3.70E-01	9.90E-01
negative regulation of neuron apoptotic process	3	1.2	3.70E-01	9.90E-01
regulation of cell shape	3	1.2	3.90E-01	9.90E-01
protein stabilization	3	1.2	3.90E-01	9.90E-01

negative regulation of gene expression	3	1.2	3.90E-01	9.90E-01
response to heat	2	0.8	3.90E-01	9.90E-01
defense response to Gram-negative bacterium	2	0.8	4.00E-01	1.00E+00
outflow tract morphogenesis	2	0.8	4.00E-01	1.00E+00
positive regulation of transcription from RNA polymerase II promoter	10	4	4.10E-01	1.00E+00
T cell receptor signaling pathway	2	0.8	4.10E-01	1.00E+00
positive regulation of B cell proliferation	2	0.8	4.20E-01	1.00E+00
positive regulation of fat cell differentiation	2	0.8	4.40E-01	1.00E+00
histone H3 acetylation	2	0.8	4.40E-01	1.00E+00
cellular response to interleukin-1	2	0.8	4.40E-01	1.00E+00
wound healing	2	0.8	4.40E-01	1.00E+00
neuromuscular process controlling balance	2	0.8	4.40E-01	1.00E+00
cell chemotaxis	2	0.8	4.50E-01	1.00E+00
protein homotetramerization	2	0.8	4.50E-01	1.00E+00
negative regulation of protein phosphorylation	2	0.8	4.50E-01	1.00E+00
circadian regulation of gene expression	2	0.8	4.60E-01	1.00E+00
activation of cysteine-type endopeptidase activity involved in apoptotic process	2	0.8	4.60E-01	1.00E+00
cell adhesion	4	1.6	4.90E-01	1.00E+00
chloride transmembrane transport	2	0.8	4.90E-01	1.00E+00
defense response to bacterium	2	0.8	4.90E-01	1.00E+00
positive regulation of JNK cascade	2	0.8	5.00E-01	1.00E+00
positive regulation of ERK1 and ERK2 cascade	3	1.2	5.10E-01	1.00E+00
positive regulation of protein phosphorylation	2	0.8	5.10E-01	1.00E+00
positive regulation of osteoblast differentiation	2	0.8	5.20E-01	1.00E+00
lipid catabolic process	2	0.8	5.20E-01	1.00E+00
activation of MAPK activity	2	0.8	5.30E-01	1.00E+00
cellular response to lipopolysaccharide	2	0.8	5.30E-01	1.00E+00
positive regulation of transcription, DNA-templated	5	2	5.30E-01	1.00E+00
positive regulation of protein binding	2	0.8	5.40E-01	1.00E+00
skeletal system development	2	0.8	5.50E-01	1.00E+00
protein autophosphorylation	3	1.2	5.50E-01	1.00E+00
cell-matrix adhesion	2	0.8	5.60E-01	1.00E+00
transcription from RNA polymerase II promoter	3	1.2	5.80E-01	1.00E+00
ER to Golgi vesicle-mediated transport	2	0.8	5.80E-01	1.00E+00
positive regulation of protein kinase B signaling	2	0.8	5.90E-01	1.00E+00
positive regulation of cell migration	3	1.2	5.90E-01	1.00E+00
positive regulation of cell proliferation	4	1.6	6.10E-01	1.00E+00
positive regulation of angiogenesis	2	0.8	6.30E-01	1.00E+00
cell redox homeostasis	2	0.8	6.30E-01	1.00E+00
cell proliferation	3	1.2	6.60E-01	1.00E+00
response to hypoxia	2	0.8	6.60E-01	1.00E+00
positive regulation of NF-kappaB transcription factor activity	2	0.8	6.60E-01	1.00E+00
metabolic process	2	0.8	6.90E-01	1.00E+00
negative regulation of cell growth	2	0.8	7.10E-01	1.00E+00

regulation of transcription from RNA polymerase II promoter	4	1.6	7.30E-01	1.00E+00
proteolysis	2	0.8	7.60E-01	1.00E+00
protein transport	2	0.8	7.60E-01	1.00E+00
intracellular signal transduction	4	1.6	8.00E-01	1.00E+00
protein folding	2	0.8	8.00E-01	1.00E+00
negative regulation of transcription from RNA polymerase II promoter	5	2	8.00E-01	1.00E+00
cilium assembly	2	0.8	8.10E-01	1.00E+00
negative regulation of cell proliferation	3	1.2	8.10E-01	1.00E+00
negative regulation of transcription, DNA-templated	3	1.2	8.30E-01	1.00E+00
angiogenesis	2	0.8	8.50E-01	1.00E+00
protein ubiquitination involved in ubiquitin-dependent protein catabolic process	2	0.8	8.50E-01	1.00E+00
multicellular organism development	3	1.2	8.80E-01	1.00E+00
protein ubiquitination	2	0.8	8.80E-01	1.00E+00
regulation of transcription, DNA-templated	5	2	9.50E-01	1.00E+00

Supplemental Table 4

P1C5/H3N8 vs. R(low)/H3N8 Gene Downregulated DEG DAVID Gene Ontology				
Term	**Count**	**%**	**P-Value**	**Benjamini**
cilium assembly	6	5.7	1.60E-04	4.10E-02
inner dynein arm assembly	3	2.8	1.10E-03	1.40E-01
intraciliary transport	3	2.8	1.40E-03	1.20E-01
sperm motility	3	2.8	1.20E-02	5.60E-01
retina development in camera-type eye	3	2.8	1.30E-02	5.10E-01
determination of left/right symmetry	3	2.8	1.70E-02	5.40E-01
prostaglandin metabolic process	2	1.9	1.90E-02	5.30E-01
cilium movement involved in cell motility	2	1.9	1.90E-02	5.30E-01
neural tube patterning	2	1.9	2.60E-02	5.90E-01
intraciliary transport involved in cilium morphogenesis	2	1.9	3.80E-02	6.90E-01
intraciliary retrograde transport	2	1.9	3.80E-02	6.90E-01
epithelial cilium movement	2	1.9	4.40E-02	7.10E-01
motile cilium assembly	2	1.9	5.00E-02	7.20E-01
GTP biosynthetic process	2	1.9	5.00E-02	7.20E-01
CTP biosynthetic process	2	1.9	5.00E-02	7.20E-01
UTP biosynthetic process	2	1.9	5.00E-02	7.20E-01
aorta development	2	1.9	8.10E-02	8.50E-01
coronary vasculature development	2	1.9	9.30E-02	8.70E-01
lens development in camera-type eye	2	1.9	1.40E-01	9.40E-01
spermatid development	2	1.9	2.00E-01	9.80E-01
microtubule-based movement	2	1.9	2.40E-01	9.90E-01
Notch signaling pathway	2	1.9	2.60E-01	9.90E-01
Golgi organization	2	1.9	2.90E-01	9.90E-01
mitotic nuclear division	2	1.9	3.10E-01	9.90E-01

heart development	2	1.9	4.70E-01	1.00E+00
cell proliferation	2	1.9	5.10E-01	1.00E+00
positive regulation of transcription, DNA-templated	2	1.9	7.10E-01	1.00E+00
intracellular signal transduction	2	1.9	7.40E-01	1.00E+00
positive regulation of transcription from RNA polymerase II promoter	2	1.9	9.30E-01	1.00E+00

Supplemental Table 5

Mock/H3N8 vs. P1C5/H3N8 Downregulated DEG DAVID Gene Ontology				
Term	**Count**	**%**	**P-Value**	**Benjamini**
toll-like receptor signaling pathway	4	5.6	2.50E-04	8.70E-02
inflammatory response	6	8.3	8.50E-04	1.40E-01
MyD88-dependent toll-like receptor signaling pathway	3	4.2	2.60E-03	2.70E-01
tolerance induction to lipopolysaccharide	2	2.8	1.20E-02	6.60E-01
response to virus	3	4.2	1.40E-02	6.30E-01
smooth muscle cell migration	2	2.8	1.80E-02	6.60E-01
innate immune response	4	5.6	2.30E-02	7.00E-01
detection of lipopolysaccharide	2	2.8	2.40E-02	6.60E-01
positive regulation of keratinocyte migration	2	2.8	3.00E-02	7.00E-01
skeletal muscle contraction	2	2.8	3.50E-02	7.30E-01
positive regulation of chemokine production	2	2.8	4.10E-02	7.50E-01
positive regulation of wound healing	2	2.8	4.70E-02	7.70E-01
negative regulation of smooth muscle cell proliferation	2	2.8	6.40E-02	8.40E-01
negative regulation of interleukin-6 production	2	2.8	6.40E-02	8.40E-01
negative regulation of tumor necrosis factor production	2	2.8	8.60E-02	9.00E-01
cardiac muscle contraction	2	2.8	8.60E-02	9.00E-01
negative regulation of extrinsic apoptotic signaling pathway via death domain receptors	2	2.8	8.60E-02	9.00E-01
positive regulation of smooth muscle cell proliferation	2	2.8	8.60E-02	9.00E-01
endodermal cell differentiation	2	2.8	9.20E-02	9.00E-01
protein homotrimerization	2	2.8	9.20E-02	9.00E-01
positive regulation of I-kappaB kinase/NF-kappaB signaling	3	4.2	9.60E-02	9.00E-01
negative regulation of endothelial cell apoptotic process	2	2.8	9.70E-02	8.90E-01

Supplemental Table 6

Mock/H3N8 vs. Mock/Rlow				
Downregulated KEGG Pathways				
Term	**Count**	**%**	**P-Value**	**Benjamini**
Cardiac muscle contraction	4	5.1	1.20E-02	4.20E-01
Oxidative phosphorylation	5	6.3	1.30E-02	2.60E-01
Focal adhesion	5	6.3	5.90E-02	6.00E-01
ECM-receptor interaction	3	3.8	1.20E-01	7.50E-01
Toll-like receptor signaling pathway	3	3.8	1.40E-01	7.50E-01
Porphyrin and chlorophyll metabolism	2	2.5	1.80E-01	7.80E-01
Phagosome	3	3.8	2.60E-01	8.60E-01
Protein processing in endoplasmic reticulum	3	3.8	3.30E-01	9.00E-01
PPAR signaling pathway	2	2.5	3.80E-01	9.10E-01
Lysosome	2	2.5	6.00E-01	9.80E-01
Cytokine-cytokine receptor interaction	2	2.5	7.00E-01	9.90E-01
Regulation of actin cytoskeleton	2	2.5	7.70E-01	1.00E+00
Metabolic pathways	8	10	7.90E-01	1.00E+00

Supplemental Table 7

Mock/H3N8 vs. P1C5/H3N8				
Downregulated KEGG Pathways				
Term	**Count**	**%**	**P-Value**	**Benjamini**
Cardiac muscle contraction	4	5.6	5.90E-03	2.30E-01
NOD-like receptor signaling pathway	3	4.2	2.50E-02	4.40E-01
Oxidative phosphorylation	4	5.6	3.40E-02	4.00E-01
Salmonella infection	3	4.2	6.30E-02	5.20E-01
Phenylalanine metabolism	2	2.8	8.40E-02	5.50E-01
Toll-like receptor signaling pathway	3	4.2	9.40E-02	5.20E-01
Phagosome	3	4.2	1.80E-01	7.10E-01
Tyrosine metabolism	2	2.8	1.80E-01	6.70E-01
Influenza A	3	4.2	1.90E-01	6.50E-01
Cytosolic DNA-sensing pathway	2	2.8	2.20E-01	6.80E-01
Cytokine-cytokine receptor interaction	3	4.2	2.30E-01	6.60E-01
PPAR signaling pathway	2	2.8	3.10E-01	7.50E-01
Metabolic pathways	7	9.7	6.80E-01	9.80E-01
Focal adhesion	2	2.8	6.90E-01	9.80E-01
Neuroactive ligand-receptor interaction	2	2.8	8.20E-01	9.90E-01

Supplemental Table 8

Mock/H3N8 vs. Rlow/H3N8				
Upregulated KEGG Pathways				
Term	Count	%	P-Value	Benjamini
Phagosome	12	4.6	1.30E-04	1.00E-02
Cell adhesion molecules (CAMs)	11	4.2	2.30E-04	9.30E-03
Intestinal immune network for IgA production	6	2.3	7.20E-04	2.00E-02
Lysosome	10	3.8	1.10E-03	2.20E-02
Jak-STAT signaling pathway	10	3.8	1.50E-03	2.40E-02
Toll-like receptor signaling pathway	8	3.1	3.30E-03	4.50E-02
Protein processing in endoplasmic reticulum	10	3.8	7.50E-03	8.40E-02
Cytokine-cytokine receptor interaction	10	3.8	7.50E-03	8.40E-02
Influenza A	9	3.5	1.10E-02	1.00E-01
Herpes simplex infection	8	3.1	5.00E-02	3.70E-01
Nicotinate and nicotinamide metabolism	3	1.2	1.30E-01	6.90E-01
VEGF signaling pathway	4	1.5	1.50E-01	7.10E-01
Cytosolic DNA-sensing pathway	3	1.2	2.50E-01	8.60E-01
ECM-receptor interaction	4	1.5	2.50E-01	8.40E-01
Focal adhesion	7	2.7	2.70E-01	8.50E-01
Glycosaminoglycan degradation	2	0.8	3.50E-01	9.10E-01
Phosphatidylinositol signaling system	4	1.5	3.70E-01	9.10E-01
RIG-I-like receptor signaling pathway	3	1.2	3.80E-01	9.00E-01
Protein export	2	0.8	4.10E-01	9.10E-01
Porphyrin and chlorophyll metabolism	2	0.8	4.50E-01	9.30E-01
Drug metabolism - other enzymes	2	0.8	4.70E-01	9.20E-01
Cysteine and methionine metabolism	2	0.8	5.50E-01	9.60E-01
ErbB signaling pathway	3	1.2	5.50E-01	9.50E-01
MAPK signaling pathway	6	2.3	5.70E-01	9.50E-01
ABC transporters	2	0.8	5.90E-01	9.50E-01
N-Glycan biosynthesis	2	0.8	6.70E-01	9.70E-01
mTOR signaling pathway	2	0.8	7.00E-01	9.80E-01
Arachidonic acid metabolism	2	0.8	7.00E-01	9.70E-01
Apoptosis	2	0.8	7.20E-01	9.80E-01
PPAR signaling pathway	2	0.8	7.60E-01	9.80E-01
Insulin signaling pathway	3	1.2	7.70E-01	9.80E-01
p53 signaling pathway	2	0.8	7.70E-01	9.80E-01
Endocytosis	5	1.9	7.70E-01	9.80E-01
FoxO signaling pathway	3	1.2	7.90E-01	9.80E-01
Adipocytokine signaling pathway	2	0.8	7.90E-01	9.80E-01
Regulation of actin cytoskeleton	4	1.5	8.00E-01	9.80E-01
Inositol phosphate metabolism	2	0.8	8.10E-01	9.80E-01
Progesterone-mediated oocyte maturation	2	0.8	8.40E-01	9.80E-01
Purine metabolism	3	1.2	8.80E-01	9.90E-01
Pyrimidine metabolism	2	0.8	8.80E-01	9.90E-01
Calcium signaling pathway	3	1.2	9.00E-01	9.90E-01
Insulin resistance	2	0.8	9.00E-01	9.90E-01
Vascular smooth muscle contraction	2	0.8	9.10E-01	9.90E-01
Wnt signaling pathway	2	0.8	9.50E-01	1.00E+00
Metabolic pathways	18	6.9	9.80E-01	1.00E+00
Neuroactive ligand-receptor interaction	2	0.8	1.00E+00	1.00E+00

Mock/H3N8 vs. Rlow/H3N8				
Downregulated KEGG Pathways				
Term	Count	%	P-Value	Benjamini
Cardiac muscle contraction	5	1.7	5.10E-03	3.00E-01
Purine metabolism	7	2.4	7.60E-03	2.30E-01
Oxidative phosphorylation	6	2.1	1.10E-02	2.20E-01
Metabolic pathways	21	7.3	1.40E-02	2.10E-01
Biosynthesis of antibiotics	7	2.4	1.90E-02	2.30E-01
Pyrimidine metabolism	4	1.4	8.00E-02	6.20E-01
Drug metabolism - cytochrome P450	2	0.7	2.80E-01	9.60E-01
Metabolism of xenobiotics by cytochrome P450	2	0.7	3.10E-01	9.60E-01
Cysteine and methionine metabolism	2	0.7	3.20E-01	9.50E-01
Glycine, serine and threonine metabolism	2	0.7	3.40E-01	9.40E-01
Adrenergic signaling in cardiomyocytes	3	1	3.80E-01	9.50E-01
Fatty acid metabolism	2	0.7	4.10E-01	9.50E-01
Valine, leucine and isoleucine degradation	2	0.7	4.10E-01	9.50E-01
Arachidonic acid metabolism	2	0.7	4.50E-01	9.60E-01
PPAR signaling pathway	2	0.7	5.10E-01	9.70E-01
ECM-receptor interaction	2	0.7	5.80E-01	9.80E-01
TGF-beta signaling pathway	2	0.7	5.90E-01	9.80E-01
Tight junction	2	0.7	6.00E-01	9.80E-01
Insulin resistance	2	0.7	6.80E-01	9.90E-01
Vascular smooth muscle contraction	2	0.7	7.00E-01	9.90E-01
Cell adhesion molecules (CAMs)	2	0.7	7.30E-01	9.90E-01
Cell cycle	2	0.7	7.30E-01	9.90E-01
Insulin signaling pathway	2	0.7	7.50E-01	9.90E-01
FoxO signaling pathway	2	0.7	7.60E-01	9.90E-01
Calcium signaling pathway	2	0.7	8.60E-01	1.00E+00
Focal adhesion	2	0.7	8.90E-01	1.00E+00
Endocytosis	2	0.7	9.30E-01	1.00E+00
Neuroactive ligand-receptor interaction	2	0.7	9.60E-01	1.00E+00

Supplemental Table 9

P1C5/H3N8 vs. Rlow/H3N8 Upregulated KEGG Pathways				
Term	Count	%	P-Value	Benjamini
Cytokine-cytokine receptor interaction	16	6.3	1.90E-06	1.40E-04
Toll-like receptor signaling pathway	11	4.3	2.80E-05	1.10E-03
Intestinal immune network for IgA production	7	2.8	8.40E-05	2.10E-03
Jak-STAT signaling pathway	12	4.7	9.60E-05	1.80E-03
Cell adhesion molecules (CAMs)	11	4.3	2.90E-04	4.40E-03
Phagosome	10	4	2.80E-03	3.40E-02
Influenza A	10	4	3.80E-03	4.10E-02
PPAR signaling pathway	6	2.4	1.40E-02	1.30E-01
Lysosome	8	3.2	1.80E-02	1.40E-01
Protein processing in endoplasmic reticulum	8	3.2	6.60E-02	4.00E-01
Herpes simplex infection	7	2.8	1.30E-01	6.20E-01
VEGF signaling pathway	4	1.6	1.60E-01	6.80E-01
Cytosolic DNA-sensing pathway	3	1.2	2.60E-01	8.20E-01
NOD-like receptor signaling pathway	3	1.2	2.60E-01	8.20E-01
ECM-receptor interaction	4	1.6	2.70E-01	8.10E-01
Fatty acid biosynthesis	2	0.8	2.80E-01	8.10E-01
Fatty acid metabolism	3	1.2	2.90E-01	8.10E-01
Focal adhesion	7	2.8	3.00E-01	7.90E-01
Nitrogen metabolism	2	0.8	3.40E-01	8.30E-01
Glycosaminoglycan biosynthesis	2	0.8	3.80E-01	8.50E-01
RIG-I-like receptor signaling pathway	3	1.2	3.90E-01	8.50E-01
MAPK signaling pathway	7	2.8	4.20E-01	8.60E-01
Protein export	2	0.8	4.20E-01	8.50E-01
Regulation of actin cytoskeleton	6	2.4	4.40E-01	8.50E-01
Porphyrin and chlorophyll metabolism	2	0.8	4.60E-01	8.60E-01
Adipocytokine signaling pathway	3	1.2	4.70E-01	8.50E-01
Salmonella infection	3	1.2	4.80E-01	8.60E-01
Alanine, aspartate and glutamate metabolism	2	0.8	5.50E-01	8.90E-01
Cysteine and methionine metabolism	2	0.8	5.60E-01	8.90E-01
Fatty acid degradation	2	0.8	5.60E-01	8.90E-01
ErbB signaling pathway	3	1.2	5.70E-01	8.90E-01
Pyrimidine metabolism	3	1.2	6.30E-01	9.20E-01
Sphingolipid metabolism	2	0.8	7.10E-01	9.50E-01
Arachidonic acid metabolism	2	0.8	7.10E-01	9.50E-01
Apoptosis	2	0.8	7.30E-01	9.50E-01
Endocytosis	4	1.6	9.10E-01	1.00E+00
Insulin signaling pathway	2	0.8	9.40E-01	1.00E+00
Oxidative phosphorylation	2	0.8	9.40E-01	1.00E+00
Wnt signaling pathway	2	0.8	9.50E-01	1.00E+00
Ubiquitin mediated proteolysis	2	0.8	9.50E-01	1.00E+00
Purine metabolism	2	0.8	9.80E-01	1.00E+00
Metabolic pathways	16	6.3	1.00E+00	1.00E+00

P1C5/H3N8 vs. Rlow/H3N8 Downregulated KEGG Pathways			
Term	Count	%	P-Value
Metabolic pathways	10	9.4	6.60E-02
Drug metabolism - cytochrome P450	2	1.9	1.30E-01
Retinol metabolism	2	1.9	1.40E-01
Tyrosine metabolism	2	1.9	1.50E-01
Fatty acid degradation	2	1.9	1.60E-01
Purine metabolism	3	2.8	1.70E-01
Valine, leucine and isoleucine degradation	2	1.9	2.00E-01
Biosynthesis of antibiotics	3	2.8	2.40E-01
Pyrimidine metabolism	2	1.9	3.60E-01
Phagosome	2	1.9	4.70E-01
RNA transport	2	1.9	4.90E-01

Supplemental Table 10

Mock/H3N8 vs. R(low)/H3N8 > 2 log2 FC Sig DEG				
Gene ID	Gene	Protein Name	Log2 FC	q value
ENSGALG00000019478	ENSGALG00000019478	Uncharacterized protein	#NAME?	0.0048
ENSGALG00000023458	ENSGALG00000023458	Uncharacterized protein	#NAME?	0.0048
ENSGALG00000023937	ENSGALG00000023937	Uncharacterized protein	#NAME?	0.0263
ENSGALG00000025628	ENSGALG00000025628	Uncharacterized protein	2.465	0.0048
ENSGALG00000017790	5S_rRNA	5S ribosomal RNA	2.019	0.0442
ENSGALG00000024292	MEF2B	myocyte enhancer factor 2B	2.009	0.0212

Mock/H3N8 vs. R(low)/H3N8 < -2 log2 FC Sig DEG				
Gene ID	**Gene**	**Protein Name**	**Log2 FC**	**q value**
ENSGALG00000012997	DNAH5	dynein, axonemal, heavy chain 5	-2.007	0.013
ENSGALG00000005500	HSBP1	heat shock factor-binding protein 1	-2.007	0.005
ENSGALG00000002553	PCDH1	Gallus gallus protocadherin 1 (PCDH1), mRNA	-2.013	0.021
ENSGALG00000019164	CXORF30		-2.019	0.021
ENSGALG00000000451	ENSGALG00000000451	Uncharacterized protein	-2.031	0.023
ENSGALG00000019240	GGA.46624		-2.037	0.005
ENSGALG00000013247	CCDC83	coiled-coil domain containing 83	-2.037	0.008
ENSGALG00000008109	CDHR3	cadherin related family member 3	-2.040	0.013
ENSGALG00000004121	GPR23	lysophosphatidic acid receptor 4	-2.084	0.025
ENSGALG00000015214	AKD1		-2.089	0.027
ENSGALG00000000774	TLR21	Toll-like receptor 21 precursor	-2.100	0.018
ENSGALG00000005601	ENSGALG00000005601	Uncharacterized protein	-2.104	0.005
ENSGALG00000008910	CERKL	ceramide kinase like	-2.110	0.005
ENSGALG00000010712	NDUFA4		-2.112	0.005
ENSGALG00000011277	PLEKHG7	pleckstrin homology and RhoGEF domain containing G7	-2.114	0.008
ENSGALG00000006048	FAM64A	family with sequence similarity 64 member A	-2.122	0.011
ENSGALG00000017226	CCDC67	coiled-coil domain containing 67	-2.129	0.005
ENSGALG00000000287	AGPAT1		-2.151	0.020
ENSGALG00000008879	CCDC39		-2.163	0.011
ENSGALG00000022869	TMEM200C		-2.187	0.018
ENSGALG00000020920	ENSGALG00000020920	Uncharacterized protein	-2.190	0.036
ENSGALG00000017190	KIAA1377	centrosomal protein 126kDa	-2.201	0.028
ENSGALG00000000447	KCNA3	potassium voltage-gated channel subfamily A member 3	-2.230	0.013
ENSGALG00000008519	NR2C2	nuclear receptor subfamily 2 group C member 2	-2.248	0.022
ENSGALG00000003239	ENSGALG00000003239	Uncharacterized protein	-2.258	0.005
ENSGALG00000017023	NEK5	NIMA-related kinase 5	-2.301	0.014
ENSGALG00000006841	POLR2L	polymerase (RNA) II (DNA directed) polypeptide L, 7.6kDa	-2.314	0.046
ENSGALG00000022714	C11ORF75		-2.333	0.045
ENSGALG00000003615	ENSGALG00000003615	Uncharacterized protein	-2.339	0.014
ENSGALG00000015272	ENSGALG00000015272	Uncharacterized protein	-2.356	0.015
ENSGALG00000011410	ENSGALG00000011410	Uncharacterized protein	-2.395	0.014
ENSGALG00000015358	MYH15	myosin heavy chain, cardiac muscle isoform	-2.440	0.035
ENSGALG00000009732	PCDH18	protocadherin 18	-2.445	0.005
ENSGALG00000015164	PCSK5	proprotein convertase subtilisin/kexin type 5	-2.468	0.008
ENSGALG00000022741	ENSGALG00000022741	Uncharacterized protein	-2.473	0.005
ENSGALG00000009620	FSIP1		-2.507	0.048
ENSGALG00000016962	ENSGALG00000016962	Uncharacterized protein	-2.537	0.005
ENSGALG00000007537	INCENP	inner centromere protein	-2.567	0.005
ENSGALG00000016281	DMD	dystrophin	-2.575	0.005
ENSGALG00000015329	ENSGALG00000015329	Uncharacterized protein	-2.608	0.005

ENSGALG00000008848	SOX2	Transcription factor SOX-2	-2.690	0.028
ENSGALG00000002552	COL1A1	Gallus gallus collagen, type III, alpha 1 (COL3A1), mRNA	-2.692	0.005
ENSGALG00000021544	ENSGALG00000021544	Uncharacterized protein	-2.703	0.015
ENSGALG00000020228	ENSGALG00000020228	Uncharacterized protein	-2.727	0.015
ENSGALG00000023137	SPTSSA		-2.788	0.005
ENSGALG00000023229	H2B-VIII		-3.031	0.011
ENSGALG00000022740	ENSGALG00000022740	Uncharacterized protein	-3.069	0.047
ENSGALG00000020982	BPIFB3	BPI fold containing family B member 3	-3.097	0.005
ENSGALG00000014843	TPD52L1	Gallus gallus tumor protein D52-like 1 (TPD52L1), mRNA	-3.372	0.005
ENSGALG00000005843	EEF1A2	eukaryotic translation elongation factor 1 alpha 2	-3.374	0.005
ENSGALG00000006010	FAM103A1	protein FAM103A1	-3.543	0.005
ENSGALG00000015741	ENSGALG00000015741	Uncharacterized protein	-4.074	0.026
ENSGALG00000013193	IRX2	iroquois-class homeodomain protein IRX-2	-4.435	0.045

Supplemental Table 11

Mock/H3N8 vs. P1C5/H3N8 > 2 log2 Fold Change (FC) Significant DEG				
Gene ID	**Gene**	**Protein Name**	**Log2 FC**	**q value**
ENSGALG00000009262	FGB	fibrinogen beta chain	3.331	0.010
ENSGALG00000003519	CHIA	chitinase, acidic	2.999	0.010
ENSGALG00000005278	CDH11	cadherin 11	2.809	0.039
ENSGALG00000025628	ENSGALG00000025628	Uncharacterized protein	2.417	0.010
ENSGALG00000016682	CRISP1	Cysteine Rich Secretory Protein 1	2.173	0.010
Mock/H3N8 vs. P1C5/H3N8 < -2 log2 Fold Change (FC) Significant DEG				
Gene ID	**Gene**	**Protein Name**	**Log2 FC**	**q value**
ENSGALG00000023521	ADRA2A	adrenoceptor alpha 2A	-5.373	0.035
ENSGALG00000002553	PCDH1	protocadherin 1	-3.658	0.010
ENSGALG00000015446	POU2F1	POU class 2 homeobox 1	-3.056	0.010
ENSGALG00000006010	FAM103A1	family with sequence similarity 103 member A1	-3.021	0.010
ENSGALG00000020228	ENSGALG00000020228	Uncharacterized protein	-2.812	0.010
ENSGALG00000019061	MMP1	matrix metallopeptidase 1	-2.809	0.010
ENSGALG00000004167	SRGN	serglycin	-2.705	0.010
ENSGALG00000006841	POLR2L	polymerase (RNA) II (DNA directed) polypeptide L 7.6kDa	-2.621	0.027
ENSGALG00000020920	ENSGALG00000020920	Uncharacterized protein	-2.619	0.010
ENSGALG00000004159	GGA.38350		-2.585	0.050
ENSGALG00000014997	PLA1A	phospholipase A1 member A	-2.455	0.044
ENSGALG00000004041	ENSGALG00000004041	Uncharacterized protein	-2.448	0.010
ENSGALG00000016112	NOV	nephroblastoma overexpressed gene	-2.441	0.010
ENSGALG00000006295	ENSGALG00000006295	Uncharacterized protein	-2.368	0.010
ENSGALG00000014412	CSTA	cystatin A	-2.356	0.010
ENSGALG00000000774	TLR21	similar to Toll-like receptor 21	-2.346	0.010

ENSGALG00000016782	IL1R2	interleukin 1 receptor type II	-2.313	0.010
ENSGALG00000021490	GPR97	G protein-coupled receptor 97	-2.313	0.022
ENSGALG00000014182	ADORA2B	adenosine A2b receptor	-2.278	0.044
ENSGALG00000000534	IL-1BETA	il-1beta	-2.262	0.010
ENSGALG00000017033	MRPS31	mitochondrial ribosomal protein S31	-2.251	0.010
ENSGALG00000006992	MMP9	matrix metalloproteinase-9 precursor	-2.224	0.010
ENSGALG00000019657	GGA.52241	Uncharacterized protein	-2.157	0.039
ENSGALG00000005599	ENSGALG00000005599	Uncharacterized protein	-2.140	0.039
ENSGALG00000006326	MIF	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	-2.139	0.027
ENSGALG00000016693	ENSGALG00000016693	Uncharacterized protein	-2.120	0.017
ENSGALG00000017214	HEPHL1	hephaestin like 1	-2.101	0.039
ENSGALG00000023340	ENSGALG00000023340	Uncharacterized protein	-2.054	0.010
ENSGALG00000022740	ENSGALG00000022740	Uncharacterized protein	-2.044	0.010
ENSGALG00000002234	UNC13D	unc-13 homolog D (C. elegans)	-2.034	0.010
ENSGALG00000003615	ENSGALG00000003615	Uncharacterized protein	-2.024	0.017
ENSGALG00000007463	HBAD	Gallus gallus alpha-D-globin (HBAD), mRNA	-2.016	0.039

Supplemental Table 12

Mock/R(low) vs. H3N8/R(low) > 2 log2 Fold Change (FC) Significant DEG				
Gene ID	**Gene**	**Protein Name**	**Log2 FC**	**q value**
ENSGALG00000006593	CLDN18	claudin 18	#NAME?	0.037
ENSGALG00000012928	ENSGALG00000012928	Uncharacterized protein	inf	0.037
ENSGALG00000005278	CDH11	cadherin-11 precursor	6.757	0.037
ENSGALG00000009732	PCDH18	protocadherin 18	2.870	0.037
ENSGALG00000007945	CRYAB	Alpha-crystallin B chain	2.707	0.037
ENSGALG00000011469	IGFBP2	Gallus gallus insulin-like growth factor binding protein 2, 36kDa (IGFBP2), mRNA	2.582	0.037
ENSGALG00000005172	ENSGALG00000005172	Uncharacterized protein	2.237	0.037
ENSGALG00000002435	DUOX2	Dual Oxidase 2	2.053	0.037
Mock/R(low) vs. H3N8/R(low) < -2 log2 Fold Change (FC) Significant DEG				
Gene ID	**Gene**	**Protein Name**	**Log2 FC**	**q value**
ENSGALG00000014843	TPD52L1	Gallus gallus tumor protein D52-like 1 (TPD52L1), mRNA	-2.334	0.037
ENSGALG00000013811	NCAPH2	non-SMC condensin II complex subunit H2	-2.309	0.037
ENSGALG00000016171	PTK2	Focal adhesion kinase 1	-2.208	0.037

Supplemental Table 13

P1C5/H3N8 vs. R(low)/H3N8 > 2 log2 Fold Change (FC) Significant DEG				
Gene ID	**Gene**	**Protein Name**	**Log2 FC**	**q value**
ENSGALG00000019061	MMP1	matrix metallopeptidase 1	3.801	0.004
ENSGALG00000006992	MMP9	matrix metallopeptidase 9	3.704	0.004
ENSGALG00000000534	IL-1BETA	interleukin 1, beta (IL1B), mRNA	3.508	0.004
ENSGALG00000011668	K60	interleukin-8 precursor	3.338	0.004
ENSGALG00000002234	UNC13D	unc-13 homolog D (C. elegans)	3.315	0.004
ENSGALG00000000081	IL4I1	L-amino-acid oxidase precursor	3.287	0.004
ENSGALG00000016782	IL1R2	interleukin 1 receptor, type II	3.253	0.004
ENSGALG00000004041	ENSGALG00000004041	Uncharacterized protein	3.208	0.004
ENSGALG00000002112	CSF3R	granulocyte colony-stimulating factor receptor	3.190	0.004
ENSGALG00000005599	ENSGALG00000005599	Uncharacterized protein	3.164	0.047
ENSGALG00000004582	MYL2	myosin regulatory light chain 2A, cardiac muscle isoform	3.042	0.004
ENSGALG00000007001	TLR4	toll-like receptor 4 precursor	3.020	0.004
ENSGALG00000008166	TLR15	toll-like receptor 15 (TLR15), mRNA	2.981	0.004
ENSGALG00000002766	MLKL	mixed lineage kinase domain-like	2.901	0.004
ENSGALG00000023818	ENSGALG00000023818	Uncharacterized protein	2.893	0.004
ENSGALG00000001905	DTX2	deltex 2, E3 ubiquitin ligase	2.875	0.004
ENSGALG00000004167	SRGN	serglycin	2.873	0.014
ENSGALG00000021142	ENSGALG00000021142	Uncharacterized protein	2.869	0.004
ENSGALG00000015666	TNIP2	TNFAIP3-interacting protein 2	2.862	0.004
ENSGALG00000010892	ANTXR2	anthrax toxin receptor 2	2.820	0.004
ENSGALG00000017214	HEPHL1	hephaestin like 1	2.779	0.035
ENSGALG00000010362	SUCNR1	succinate receptor 1	2.773	0.004
ENSGALG00000016919	IRG1	immunoresponsive 1 homolog	2.742	0.004
ENSGALG00000014412	CSTA	cystatin A	2.736	0.004
ENSGALG00000015446	POU2F1	POU domain, class 2, transcription factor 1	2.700	0.025
ENSGALG00000023370	ENSGALG00000023370	Uncharacterized protein	2.690	0.004
ENSGALG00000023933	G0S2	G0/G1switch 2	2.676	0.010
ENSGALG00000020316	IL13RA2	interleukin-13 receptor subunit alpha-2 precursor	2.658	0.004
ENSGALG00000000949	HBEGF	Proheparin-binding EGF-like growth factor Heparin-binding EGF-like growth factor	2.653	0.004
ENSGALG00000011670	IL8	interleukin 8 [Gallus gallus]	2.651	0.008
ENSGALG00000011221	AGPAT9	Glycerol-3-phosphate acyltransferase 3	2.631	0.004
ENSGALG00000004060	RGS9	regulator of G-protein signaling 9	2.621	0.004
ENSGALG00000002383	CD72	CD72 antigen	2.612	0.004
ENSGALG00000005172	ENSGALG00000005172	Uncharacterized protein	2.590	0.004
ENSGALG00000005941	ENSGALG00000005941	Uncharacterized protein	2.583	0.041
ENSGALG00000019148	CSF2RA		2.556	0.008
ENSGALG00000023855	KRT14	keratin, type I cytoskeletal 14	2.554	0.004
ENSGALG00000021340	CA9	carbonic anhydrase IX	2.552	0.004
ENSGALG00000023622	AVD	Avidin	2.551	0.004
ENSGALG00000006106	TNFRSF6B	tumor necrosis factor receptor superfamily member 6b	2.549	0.004
ENSGALG00000002118	CO6	calcium-activated potassium channel subunit beta-1	2.545	0.004
ENSGALG00000016693	ENSGALG00000016693	Uncharacterized protein	2.518	0.020
ENSGALG00000022646	ENSGALG00000022646	Uncharacterized protein	2.515	0.025
ENSGALG00000005086	PLAU	urokinase-type plasminogen activator preproprotein	2.463	0.004

ENSGALG00000000837	FKBP6	FK506 binding protein 6	2.460	0.016
ENSGALG00000010152	TSPAN8	tetraspanin-8	2.452	0.029
ENSGALG00000014585	CCLI10	C-C motif chemokine ligand 26	2.450	0.004
ENSGALG00000013568	NR4A3	nuclear receptor subfamily 4 group A member 3	2.447	0.004
ENSGALG00000003123	CNTNAP1	contactin associated protein 1	2.430	0.028
ENSGALG00000019147	CSF2RA	granulocyte-macrophage colony-stimulating factor receptor subunit alpha-like	2.416	0.024
ENSGALG00000004700	NCF2	neutrophil cytosolic factor 2	2.388	0.030
ENSGALG00000021355	TROJAN		2.386	0.030
ENSGALG00000000731	C1ORF38	thymocyte selection associated family member 2	2.349	0.025
ENSGALG00000003282	STAT5B	signal transducer and activator of transcription 5B	2.342	0.004
ENSGALG00000007454	PDCD1	programmed cell death 1	2.329	0.014
ENSGALG00000016788	IL18RAP	interleukin 18 receptor accessory protein	2.329	0.034
ENSGALG00000001710	ENSGALG00000001710	Uncharacterized protein	2.324	0.035
ENSGALG00000006375	TM4SF19	transmembrane 4 L6 family member 19	2.324	0.016
ENSGALG00000023340	ENSGALG00000023340	Uncharacterized protein	2.303	0.042
ENSGALG00000008308	BHLHE40	class E basic helix-loop-helix protein 40	2.296	0.004
ENSGALG00000003482	BHLHA15		2.283	0.004
ENSGALG00000000974	ENSGALG00000000974	Uncharacterized protein	2.277	0.034
ENSGALG00000013101	BG2		2.276	0.025
ENSGALG00000002728	SLC16A3	Monocarboxylate transporter 4	2.273	0.004
ENSGALG00000014962	FAM26F	family with sequence similarity 26 member F	2.273	0.010
ENSGALG00000019552	SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	2.262	0.004
ENSGALG00000015474	CD80	Gallus gallus CD80 molecule (CD80), mRNA	2.243	0.008
ENSGALG00000005430	ALS2CL	ALS2 C-terminal like	2.231	0.004
ENSGALG00000021573	PIK3R5	Phosphoinositide 3-kinase regulatory subunit 5	2.231	0.004
ENSGALG00000000736	MATK	megakaryocyte-associated tyrosine kinase	2.229	0.004
ENSGALG00000012550	HMOX1	heme oxygenase 1	2.224	0.004
ENSGALG00000001314	PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	2.224	0.016
ENSGALG00000013548	GZMA	granzyme A precursor	2.197	0.010
ENSGALG00000006204	ENSGALG00000006204	Uncharacterized protein	2.182	0.033
ENSGALG00000012830	IRF-4	interferon regulatory factor 4	2.154	0.004
ENSGALG00000005727	SLC7A3	solute carrier family 7 (cationic amino acid transporter, y+ system), member 3	2.152	0.004
ENSGALG00000005638	IL2RG	interleukin 2 receptor subunit gamma	2.149	0.004
ENSGALG00000010323	BATF	basic leucine zipper transcription factor, ATF-like	2.141	0.013
ENSGALG00000005069	PTGS2	Prostaglandin G/H synthase 2	2.140	0.004
ENSGALG00000013747	TAGAP	T-cell activation RhoGTPase activating protein	2.135	0.004
ENSGALG00000010171	NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	2.127	0.004
ENSGALG00000023820	OGCHI		2.123	0.020
ENSGALG00000007210	ENSGALG00000007210	Uncharacterized protein	2.111	0.004
ENSGALG00000000139	GGA.18046		2.106	0.018
ENSGALG00000015022	FYN	tyrosine-protein kinase Fyn	2.102	0.004
ENSGALG00000003196	ANG	Angiogenin	2.097	0.004

ENSGALG00000019251	LAG3		2.093	0.019
ENSGALG00000023634	ENSGALG00000023634	Uncharacterized protein	2.090	0.021
ENSGALG00000001076	LIMK1	LIM domain kinase 1	2.089	0.041
ENSGALG00000004243	ENSGALG00000004243	Uncharacterized protein	2.087	0.004
ENSGALG00000021616	MARCKSL1	MARCKS-related protein	2.082	0.008
ENSGALG00000016524	CAD	carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase	2.082	0.016
ENSGALG00000002540	RGS2	regulator of G-protein signaling 2	2.080	0.004
ENSGALG00000003578	FN1	fibronectin precursor	2.080	0.024
ENSGALG00000013698	EFHD2	EF-hand domain family member D2	2.072	0.010
ENSGALG00000009768	CCRN4L	nocturnin	2.053	0.019
ENSGALG00000002343	ENSGALG00000002343	Uncharacterized protein	2.052	0.048
ENSGALG00000016988	KIAA0226L	KIAA0226-like	2.046	0.004
ENSGALG00000002643	SELP	selectin P	2.035	0.004
ENSGALG00000003103	MST1R	macrophage-stimulating protein receptor precursor	2.027	0.004
ENSGALG00000013861	TNFAIP3	TNF alpha induced protein 3	2.010	0.004
ENSGALG00000002549	RGS1	regulator of G-protein signaling 1	2.001	0.004
ENSGALG00000012352	BLVRA	biliverdin reductase A	2.000	0.008
P1C5/H3N8 vs. R(low)/H3N8 < -2 log2 Fold Change (FC) Significant DEG				
Gene ID	**Gene**	**Protein Name**	**Log2 FC**	**q value**
ENSGALG00000009262	FGB	Fibrinogen beta chain Fibrinopeptide B Fibrinogen beta chain	-4.653	0.028
ENSGALG00000003519	CHIA		-4.561	0.004
ENSGALG00000016962	ENSGALG00000016962	Uncharacterized protein	-3.930	0.004
ENSGALG00000005278	CDH11	cadherin-11 precursor	-3.808	0.004
ENSGALG00000015329	ENSGALG00000015329	Uncharacterized protein	-3.752	0.004
ENSGALG00000016682	CRISP1		-3.376	0.004
ENSGALG00000002358	CDO1	cysteine dioxygenase type 1	-3.124	0.004
ENSGALG00000007945	CRYAB	Alpha-crystallin B chain	-2.933	0.045
ENSGALG00000003193	CRABP1	Cellular retinoic acid-binding protein 1	-2.910	0.004
ENSGALG00000007537	INCENP	inner centromere protein	-2.856	0.040
ENSGALG00000005843	EEF1A2	eukaryotic translation elongation factor 1 alpha 2	-2.840	0.004
ENSGALG00000006693	ENSGALG00000006693	Uncharacterized protein	-2.772	0.004
ENSGALG00000014843	TPD52L1	tumor protein D52-like 1 (TPD52L1), mRNA	-2.601	0.033
ENSGALG00000000184	SLC27A6	solute carrier family 27 (fatty acid transporter), member 6	-2.474	0.008
ENSGALG00000016292	COL21A1	collagen, type XXI, alpha 1	-2.368	0.014
ENSGALG00000023424	DMBT1		-2.344	0.004
ENSGALG00000005601	ENSGALG00000005601	Uncharacterized protein	-2.256	0.004
ENSGALG00000010417	ENSGALG00000010417	Uncharacterized protein	-2.243	0.042
ENSGALG00000006048	FAM64A	family with sequence similarity 64 member A	-2.223	0.004
ENSGALG00000020909	C20ORF85	chromosome 20 open reading frame 85 [Gallus gallus]	-2.201	0.046
ENSGALG00000010769	HPGD	15-hydroxyprostaglandin dehydrogenase	-2.154	0.004
ENSGALG00000011277	PLEKHG7	pleckstrin homology and RhoGEF domain containing G7	-2.133	0.034
ENSGALG00000009155	CHGB	chromogranin B	-2.089	0.008
ENSGALG00000020876	AOX2P	aldehyde oxidase 2 pseudogene	-2.075	0.004

ENSGALG00000012997	DNAH5	dynein, axonemal, heavy chain 5	-2.053	0.042
ENSGALG00000006217	S100B	S100 calcium binding protein B	-2.033	0.032
ENSGALG00000012250	GGA.45581	alcohol dehydrogenase 1C (class I), gamma polypeptide	-2.017	0.040

Supplemental Table 14

Mock/H3N8 vs. Mock/R(low) > 2 log2 Fold Change (FC) Significant DEG				
Gene ID	**Gene**	**Protein Name**	**Log2 FC**	**q value**
ENSGALG00000009317	ENSGALG00000009317	Uncharacterized protein	#NAME?	0.011
ENSGALG00000025284	SNORD89	Small nucleolar RNA SNORD89	#NAME?	0.011
Mock/H3N8 vs. Mock/R(low) < -2 log2 Fold Change (FC) Significant DEG				
Gene ID	**Gene**	**Protein Name**	**Log2 FC**	**q value**
ENSGALG00000014412	CSTA	cystatin A	-3.537	0.011
ENSGALG00000002553	PCDH1	Gallus gallus protocadherin 1 (PCDH1), mRNA	-3.530	0.011
ENSGALG00000000379	ITGB3	integrin beta-3 precursor	-3.136	0.011
ENSGALG00000017214	HEPHL1	hephaestin like 1	-3.087	0.011
ENSGALG00000015446	POU2F1	POU domain, class 2, transcription factor 1	-2.993	0.011
ENSGALG00000006010	FAM103A1	protein FAM103A1	-2.941	0.011
ENSGALG00000005278	CDH11	cadherin-11 precursor	-2.860	0.045
ENSGALG00000023370	ENSGALG00000023370	Uncharacterized protein	-2.850	0.011
ENSGALG00000016782	IL1R2	interleukin 1 receptor, type II	-2.849	0.011
ENSGALG00000013548	GZMA	granzyme A precursor	-2.785	0.038
ENSGALG00000021142	ENSGALG00000021142	Uncharacterized protein	-2.766	0.019
ENSGALG00000006054	CALCA	calcitonin gene-related peptide isoform 3 preproprotein	-2.704	0.041
ENSGALG00000004167	SRGN	serglycin	-2.575	0.011
ENSGALG00000020920	ENSGALG00000020920	Uncharacterized protein	-2.526	0.026
ENSGALG00000006841	POLR2L	polymerase (RNA) II (DNA directed) polypeptide L, 7.6kDa	-2.454	0.029
ENSGALG00000005172	ENSGALG00000005172	Uncharacterized protein	-2.438	0.011
ENSGALG00000023933	G0S2	G0/G1switch 2	-2.438	0.011
ENSGALG00000023340	ENSGALG00000023340	Uncharacterized protein	-2.397	0.029
ENSGALG00000005599	ENSGALG00000005599	Uncharacterized protein	-2.357	0.029
ENSGALG00000025698	5_8S_rRNA	5.8S ribosomal RNA	-2.345	0.011
ENSGALG00000019147	CSF2RA	granulocyte-macrophage colony-stimulating factor receptor subunit alpha-like	-2.336	0.026
ENSGALG00000017033	MRPS31	mitochondrial ribosomal protein S31	-2.248	0.038
ENSGALG00000014201	PARVB	parvin beta	-2.229	0.011
ENSGALG00000003578	FN1	fibronectin precursor	-2.155	0.011
ENSGALG00000019553	SERPINB10	Heterochromatin-associated protein MENT	-2.151	0.049
ENSGALG00000006326	MIF	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	-2.124	0.041
ENSGALG00000010362	SUCNR1	succinate receptor 1	-2.124	0.026
ENSGALG00000020228	ENSGALG00000020228	Uncharacterized protein	-2.112	0.041
ENSGALG00000002383	CD72	CD72 antigen	-2.091	0.049
ENSGALG00000019061	MMP1	matrix metallopeptidase 1	-2.054	0.011
ENSGALG00000008166	TLR15	Gallus gallus toll-like receptor 15 (TLR15), mRNA	-2.028	0.011

Chapter 4 - The potential contribution of *M. gallisepticum* neuraminidase activity to co-pathogenesis with LPAIV in vivo in the presence of the viral neuraminidase inhibitor, oseltamivir.

Methods

-Chicken Infection Experiment: To study the potential role of neuraminidase activity in the co-pathogenesis of *M. gallisepticum* and LPAIV, the viral neuraminidase inhibitor oseltamivir phosphate was utilized in an *in vivo* co-infection model. This inhibitor was used as a tool to observe any compensation by *M. gallisepticum* for the inhibited neuraminidase activity of LPAIV. As described in previous chapters, 10 SPF White Leghorn chickens per group were infected with combinations of *M. gallisepticum* R_{low}, the *M. gallisepticum* mutant P1C5, Hayflick's medium as a mock infection, and H3N8 (A/duck/Ukraine/1963) as outlined in the table below.

All chickens were infected intratracheally with 200 µl of either 1 x 10^8 CFU/mL of *M. gallisepticum* R_{low}, 5 x 10^6 $TCID_{50}$ of H3N8, or Hayflick's medium as a mock infection. All inoculum cultures of *M. gallisepticum* and H3N8 were diluted in fresh Hayflick's medium. Oseltamivir phosphate was administered at a dosage of 1 mg/kg per day, divided into 0.5 mg/mg twice per day, at a 1:1 ratio in phosphate buffered saline (PBS) [1].

Primary Infection (Day 0)	Oseltamivir Treatment (Day 2)	Secondary Infection (Day 3)	
Mock (Hayflick's medium)	1 mg/kg daily until day 6	H3N8	**Sacrifice (Day 7)**
Mock (Hayflick's medium)	Mock (PBS)	H3N8	
M. gallisepticum R_{low}	1 mg/kg daily until day 6	H3N8	
M. gallisepticum R_{low}	Mock (PBS)	H3N8	
M. gallisepticum P1C5	1 mg/kg daily until day 6	H3N8	
M. gallisepticum P1C5	Mock (PBS)	H3N8	

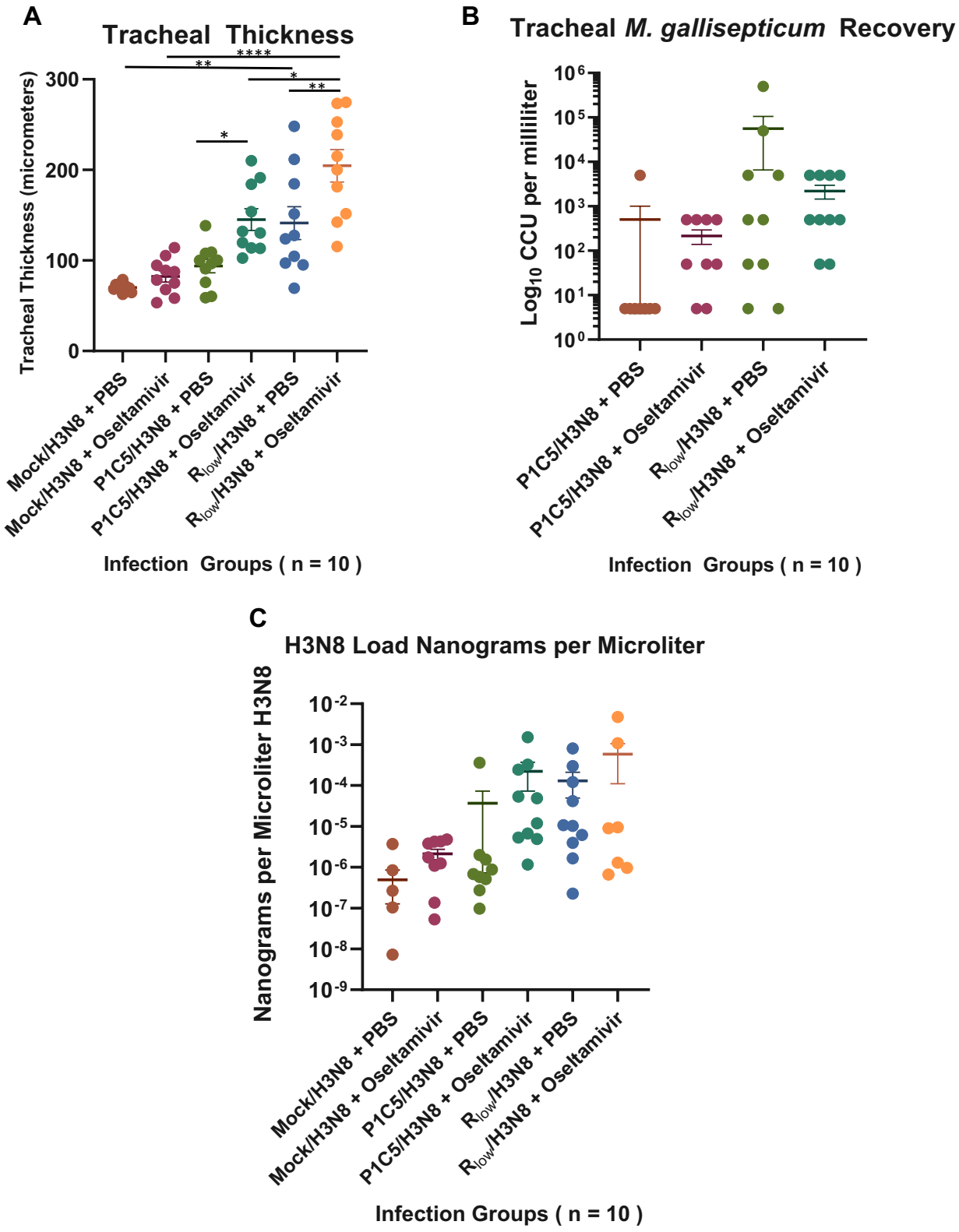
All birds were humanely sacrificed on day 7 of the experiment as described previously in this dissertation. At necropsy, gross pathologic observations were recorded and a tracheal ring from the distal end was collected for *M. gallisepticum* recovery in Hayflick's medium using 10-fold CCU dilutions. In addition, 1 mL of TriZol (Zymo Research) was washed through the lumen of each trachea to collect RNA for qPCR analysis of H3N8 load in each bird at the end of the experiment, as described earlier in this dissertation.

To investigate the potential compensatory relationship between LPAIV and *M. gallisepticum* neuraminidase activity during the course of co-infection, the *M. gallisepticum* R_{low} neuraminidase mutant, P1C5 was utilized. This mutant has been documented to be attenuated *in vivo* and unable to be recovered from infected birds at 14 days post-infection [5]. However, in conjunction with active neuraminidase activity from LPAIV H3N8, the dynamics of P1C5 may change *in vivo* allowing the mutant to persist and induce disease.

Results

Figure 1 – Tracheal mucosal thickness, bacterial, and viral load. Statistically significant differences between tracheal thickness measurements and viral loads among groups

were calculated using a one-way ANOVA with Tukey's post-hoc test and a threshold for significance at $p < 0.05$. Significant differences are indicated with **** = $p < 0.0001$, ** = $p < 0.01$, and * = $p < 0.05$. Statistically significant differncees in *M. gallisepticum* recovery among groups was calculated using a Kruskal-Wallis ANOVA On-Ranks with Dunn's Post Hoc test. No significant differences were found among experimental groups for *M. gallisepticum* or H3N8 load.


A
Tracheal Thickness
Tracheal Thickness (micrometers)
300
200
100
0
****
**
*
**
*
Mock/H3N8 + PBS
Mock/H3N8 + Oseltamivir
P1C5/H3N8 + PBS
P1C5/H3N8 + Oseltamivir
RIow/H3N8 + PBS
RIow/H3N8 + Oseltamivir
Infection Groups (n = 10)
B
Tracheal M. gallisepticum Recovery
Log10 CCU per milliliter
10^6
10^5
10^4
10^3
10^2
10^1
10^0
P1C5/H3N8 + PBS
P1C5/H3N8 + Oseltamivir
RIow/H3N8 + PBS
RIow/H3N8 + Oseltamivir
Infection Groups (n = 10)
C
H3N8 Load Nanograms per Microliter
Nanograms per Microliter H3N8
10-2
10-3
10-4
10-5
10-6
10-7
10-8
10-9
Mock/H3N8 + PBS
Mock/H3N8 + Oseltamivir
P1C5/H3N8 + PBS
P1C5/H3N8 + Oseltamivir
RIow/H3N8 + PBS
RIow/H3N8 + Oseltamivir
Infection Groups (n = 10)

-Tracheal Histopathology: Significant differences in tracheal mucosal thickness exist between multiple experimentally infected groups of chickens. As anticipated, tracheal mucosal thickness is significantly increased between Mock/H3N8 + PBS and *M. gallisepticum* R_{low}/H3N8 + PBS infected birds ($p < 0.01$) due to enhanced disease due to the presence of both *M. gallisepticum* and H3N8 infection (Figure 1A).

Similarly, a significant increase in tracheal thickness also exists between Mock/H3N8 + Oseltamivir and *M. gallisepticum* R_{low}/H3N8 + Oseltamivir infected chickens ($p < 0.0001$) (Figure 1A). The difference between these groups is confounded by the treatment with oseltamivir, which has been previously shown to effectively reduce viral load in AIV infected chickens [1]. Although the *M. gallisepticum* R_{low}/H3N8 + Oseltamivir infected group contains both co-pathogens, the oseltamivir treatment did not alter the pathologic response to this co-infection as originally hypothesized.

To expand upon this, *M. gallisepticum* R_{low}/H3N8 + Oseltamivir infected chickens in fact have significantly higher tracheal thickness measurements when compared to *M. gallisepticum* R_{low}/H3N8 + PBS infected chickens ($p < 0.01$) (Figure 1A). This same phenomenon exists between *M. gallisepticum* P1C5/H3N8 + Oseltamivir and *M. gallisepticum* P1C5H3N8 + PBS infected chickens ($p < 0.05$), in that oseltamivir treatment appears to increase the tracheal thickness in response to infection (Figure 1A). A significant difference also exists between tracheal thicknesses of *M. gallisepticum* R_{low}/H3N8 + Oseltamivir and *M. gallisepticum* P1C5/H3N8 + Oseltamivir infected chickens ($p < 0.05$) (Figure 1A).

-Tracheal *Mycoplasma gallisepticum* Recovery: Although no statistically significant differences in *M. gallisepticum* recovery from the trachea emerge among experimentally infected groups of chickens, noteworthy trends still exist (Figure 1B). *M. gallisepticum* recovery was most abundant in *M.* gallisepticum R_{low}/H3N8 + PBS infected chickens (Figure 1B). The remaining experimental groups which received an *M. gallisepticum* infection had similar levels of bacterial recovery from the trachea (Figure 1B).

-Tracheal LPAIV H3N8 Load: Similar to the recovery of *M. gallisepticum* from the trachea (Figure 1B), no statistically significant differences exist between H3N8 load in the trachea of experimentally infected groups of chickens. This is perhaps the most notable finding in that, as mentioned previously, oseltamivir treatment has been documented to significantly decrease AIV load in the tracheal tissue of infected chickens [1]. The data presented here indicate that oseltamivir treatment had no effect on the load of H3N8 in the tracheal lumen of experimentally infected chickens used in this model (Figure 1C).

Discussion

The failure of oseltamivir treatment to combat H3N8 infection in this chicken infection model was unexpected. The original hypothesis to be tested was that *M. gallisepticum* neuraminidase activity could effectively compensate for LPAIV neuraminidase in the presence of the inhibitor, oseltamivir. This relationship could also work in reverse, in that LPAIV neuraminidase may enhance the pathogenesis of a neuraminidase-deficient *M. gallisepticum* mutant, P1C5. However, the lack of

discernable difference in viral load between mock and oseltamivir treated, H3N8 infected chickens makes the appropriate testing of these hypotheses impossible.

Although the cause for the failure of oseltamivir to effectively reduce viral load in this model is unknown, there are multiple possibilities that may contribute to the explanation. Previously, Lee *et al*. utilized oseltamivir as a treatment for infection of chickens with LPAIV strain H9N2 (A/ Chicken/Korea/310/01) [1]. The LPAIV isolate in our model is of a different subtype; H3N8 (A/duck/Ukraine/1963). This difference could, at least in part, explain the difference in sensitivity to oseltamivir treatment *in vivo*. This H3N8 strain is, however, susceptible to oseltamivir carboxylate neuraminidase inhibition *in vitro*, as shown in Chapter 2 of this dissertation.

Another potential factor of the outcome of this experiment is the difference in original form of oseltamivir used between the two studies. Lee *et al*. utilized oseltamivir from capsules which was later suspended in PBS, whereas our model incorporated oseltamivir intended for oral suspension administration and these two drugs could have originated from different suppliers due to geographic differences [1].

Additionally, although the dosages of oseltamivir used was consistent between the two studies, the precise timing of administration could potentially contribute to changes in active bioavailability of the drug *in vivo*. Finally, viral isolation may have differed among tissues in our model. Sampling of other areas, such as ceacal tissues or the lung, may have illuminated potential differences in viral load in animals from this experiment.

Another interesting observation is the similarity between tracheal mucosal thickness (Figure 1A) and viral load (Figure 1C). Although no statistically significant

differences exist between the amount of virus among experimental groups, the groups with the most severe tracheal mucosal thickening are also the groups with the highest viral titer in the trachea and vice versa (Figures 1A and 1C). Although *M. gallisepticum* recovery was increased in *M.* gallisepticum R_{low} infected birds over *M.* gallisepticum P1C5 infected birds, the pattern seen between tracheal thickness and viral load does not apply as seamlessly to *M. gallisepticum* recovery.

These data, as well as the data presented in chapter 3 of this dissertation, indicate that the co-pathogen relationship between *M. gallisepticum* and LPAIV is not neuraminidase-dependent as originally hypothesized. Instead, as indicated by the host transcriptional response to mono- and co-infection, this pathobiological phenomenon is much more complex.

Alterations in transcription of genes related to tracheal ciliary activity, inflammatory immune signaling, and tissue remodeling all exist between mono- and co-infected chickens and likely contribute to differences in disease manifestation. Although some of these factors have been discussed in other publications, such as ciliostasis and cytokine levels observed in *M. gallisepticum* and LPAIV tracheal explants [2], the data presented in this dissertation are the first to examine these responses in the airway of the natural host during co-infection.

Examples of relevant transcriptional responses include significant alterations in TLR expression. TLR4 and TLR15 are increased in expression during co-infection with *M. gallisepticum* R_{low} and LPAIV H3N8 compared to mono-infected chickens, or chickens instead co-infected with attenuated *M. gallisepticum* P1C5 (Chapter 3). These two TLR genes were also abundantly expressed in the chicken trachea during infection

with *M. gallisepticum* R_{low} alone over the course of the first 7 days of infection [3]. TLR21 is significantly decreased in expression in chickens co-infected with *M. gallisepticum* R_{low} or P1C5 and H3N8 over birds infected with H3N8 alone (Chapter 3). This suppression of TLR21 signaling is a novel finding and correlates with increases in tracheal histopathology in those infected chickens (Chapter 3). It is possible that, during co-infection, TLR21 suppression prevents association with TLR4 signaling that contributes to an appropriate immune response [4].

KEGG pathway analysis also indicates differential effects on signaling pathway responses to co-infection. Noteworthy examples include differential effects on the Influenza A response pathway, extracellular matrix receptor signaling, metabolic pathways, phagocytosis, and TLR signaling (Chapter 3). Alterations in these processes in response to co-infection all contribute to the understanding of the mechanisms of the host response to co-infection with *M. gallisepticum* and LPAIV.

Another novel finding presented is the ability of attenuated *M. gallisepticum* mutants P1C5 and P1H9 to persist 6 to 7 days post-infection in the chicken trachea. These mutants were previously thought to be cleared early in infection due to their lack of survival to 14 days post-infection [5,6]. However, examination of their survival at a more acute time point suggests that both neuraminidase and MslA contribute to chronic persistence in the host, however are not required for short-term survival in the chicken airway. Co-infection with H3N8 has a more pronounced effect on chronic persistence of the MslA mutant P1H9 to 14 days post-infection than the neuraminidase mutant P1C5. This result contributes to the conclusion that the co-infection dynamic between these pathogens is more complex than originally anticipated.

Chapter 5 - Future Directions

The experiments described within this dissertation provide multiple avenues for future experimental exploration of *M. gallisepticum* and LPAIV co-pathogenesis. In attempt to reconcile the lack of efficacy of oseltamivir treatment in our chicken model, continued examination of dose, routes, and administration schedules can be performed on chickens infected with H3N8 alone. Additional tissues from these animals can be sampled to discern if other target tissues are more appropriate for changes in viral load due to this oseltamivir treatment.

In a similar vein, alternate neuraminidase inhibitors can be employed to replace oseltamivir in our model. Such inhibitors include zanamivir or DANA, as described in chapter 1 of this dissertation. Alternate isolates of LPAIV can also be utilized in our model. Our lab is in possession of another LPAIV isolate, H9N2 (A/turkey/Wisconsin/1/1966) (BEI Reagent Resources). This virus is of the same subtype as the LPAIV used by Sid *et al*. in co-infection of tracheal organ cultures with *M. gallisepticum* and LPAIV [2]. H9N2 is also growing in relevance due to its global presence in poultry and zoonotic potential [7].

Another area of interest is the transcriptomic response in *M. gallisepticum* during co-infection with LPAIV. Publications out of our laboratory have used RNA-sequencing to find coordinated changes in the expression of genes by *M. gallisepticum* during early time points of infection, and these coordinated changes may differ during a co-infection scenario due to alterations in the host environment [8,9]. Genomic changes may also occur in both *M. gallisepticum* and LPAIV in response to co-infection, and sequencing of

recovered isolates after co-infection may illuminate mutations relevant to adaptation to that co-pathogen relationship over time.

There are also multiple avenues of co-pathogenesis in this model that have yet to be explored. For instance, co-infection of alveolar cells *in* vitro with the 2009 pandemic influenza virus, pdm2009, and methicillin-resistant *Staphylococcus aureus* contributes to disruption in the barrier function of the alveolar cells [10]. This damage to a primary host defense, along with alterations in host immune signaling, contribute to the severely of disease. Although the avian respiratory tract is unique in its structure, a similar phenomenon may be occurring during co-infection with *M. gallisepticum* and LPAIV in other areas of the respiratory tract, such as the air sac.

The targeting of certain host immune molecules could also be a promising route of further investigation. In mice, alterations in TGF-β and IFNγ in a model of allergic airway disease effects the severity of co-infection of those mice with *Streptococcus pneumoniae* and influenza A virus [11]. Another example is the inhibition of IL-10 signaling in reducing disease exacerbation during co-infection of mice with *Mycobacterium tuberculosis* and influenza A virus [12].

Finally, TLR9 has been implicated in the co-pathogenesis of *Staphylococcus aureus* and influenza A virus [13]. This relationship in particular, is noteworthy in that the chicken TLR21, which is suppressed during co-infection with *M. gallisepticum* and LPAIV (Chapter 3), is a homolog of human TLR9 [14]. Therefore, TLR21, among others, could be a novel target host immune molecule for intervention of co-pathogenesis in our model with *M. gallisepticum* and LPAIV.

Other facets of viral and bacterial co-pathogenesis such as alterations in the host microbiome, biofilm formation, nutrient availability, and the availability of host receptors also provide potential routes of investigation within our co-pathogenesis model [15]. Other economically relevant viral pathogens, such as infectious bronchitis virus (IBV) could serve as viral co-pathogens of *M. gallisepticum* for other novel mechanisms of co-infection in chickens [16].

Both *M. gallisepticum* and LPAIV are pathogens of great concern to the poultry industry, with a foremost impact on small-scale backyard flocks. Farmers and hobbists keeping these smaller flocks likely do not have access to the same surveillance and prevention measures as large broiler or layer industry operations. This scenario has the potential to become even more dangerous if the flock becomes infected by a virulent field isolate of *M. gallisepticum* that can persist to a chronic state in chickens. In addition, LPAIV isolates of sub-clinical pathogenicity could propagate within a flock without being detected.

If both pathogens infect chickens within the flock, as demonstrated by the data presented here, the resulting disease could be severe. By better understanding the host-pathogen interations of this co-pathogenesis, such as the binding substrates and host immunological response, we can aid in the development of prevention and treatment efforts. This has the potential to greatly benefit the poultry industry, in that it could prevent drastic economic losses to backyard poultry farmers due to exacerbated disease during *M. gallisepticum* and LPAIV co-pathogenesis. In addition, these host-pathogen dynamics could contribute to other bacterial and viral co-pathogen relationships in other animals or humans. In summary, the data presented in this

dissertation contribute novel and interesting findings to the community of knowledge on the co-pathogenesis of *Mycoplasma gallisepticum* and LPAIV.

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